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Program Information (Division of Cancer Treatment, NCI)

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Proceedings of the Annual Spring Meeting of the Primary Breast Cancer Therapy Group: Tamoxifen Workshop

Key Biscayne, Florida

April 28, 1976

Proceedings of the US-USSR Joint Meeting on Lung Cancer

(USSR papers only)

Moscow, USSR

March 1974

This meeting was held as part of the US-USSR Chemotherapy Agreement under the US-USSR Agreement on Cancer Research. In an effort to expand the current knowledge on lung cancer research it was agreed that the USSR papers would be published in the US and the US papers would be published in the Soviet Union.

Program Information From the Division of Cancer Treatment, National Cancer Institute

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Proceedings of the Annual Spring Meeting of the Primary Breast Cancer Therapy Group: Tamoxifen Workshop

Key Biscayne, Florida

April 28, 1976

Antiestrogenic and Antitumor Properties of Tamoxifen in Laboratory Animals[1]

V. Craig Jordan[2,3,*]

SUMMARY

This paper reviews the antiestrogenic and antitumor properties of tamoxifen (NSC-180973; ICI-46474) in the rat. In classic tests for antiestrogenic activity, tamoxifen inhibits the actions of estradiol in the rat uterus and vagina. At the cellular level, tamoxifen inhibits estrogen binding to cytoplasmic estrogen receptors, but although estrogen-receptor units are translocated to the nucleus DNA synthesis does not occur. It is suggested that tamoxifen competes for estrogen receptors in the cytoplasm and the false messenger units block the nuclear acceptors which are normally activated by estradiol-estrogen receptor complexes thereby provoking DNA synthesis. Tamoxifen inhibits the growth of some 7,12-dimethylbenz(*a*)anthracene-induced rat mammary tumors whereas others continue to grow. Estrogen-stimulated rises in plasma prolactin are only partially inhibited by tamoxifen although at the tumor level, tamoxifen completely blocks estrogen binding. There is a linear correlation ($P < 0.01$) between estrogen-receptor levels in tumor biopsies before therapy and tumor responses to 3 weeks of tamoxifen treatment (50 μg/day), ie, tumors with low levels of estrogen receptors do not respond to therapy whereas tumors with higher levels of estrogen receptors regress. It is suggested that tamoxifen antagonizes the actions of estrogen at the tumor level by blocking the estrogen-receptor mechanism thereby producing tumor regression. Therefore, estrogen-receptor measurements in tumor biopsies before therapy may be a useful predictive test for the tumor response to tamoxifen treatment.

[Cancer Treat Rep 60:1409–1419, 1976]

Harper and Walpole (1,2) first described the contrasting biologic activities of tamoxifen [*trans*-1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene] and its *cis* isomer, ICI-47,699 (fig 1). In the rat, tamoxifen is antiestrogenic since it will inhibit estrogen-stimulated rises in uterine wet weight and inhibit vaginal cornification, whereas ICI-47,699 is a full estrogen and does not antagonize the actions of estradiol. By comparison, tamoxifen is a full estrogen in the mouse with ICI-47,699 being somewhat less potent (3). Of interest though, is the observation that the administration of large doses of tamoxifen to ovariectomized mice causes the vagina to cornify for a few days but then the vagina enters a prolonged diestrus when the tissue is unable to bind or respond to exogenous estradiol (4).

In this paper the antiestrogenic properties of tamoxifen in the rat will be illustrated and a theory for the mechanism of action of the compound at the cellular level will be discussed. Since tamoxifen has a spectrum of activity in the control of human breast cancer (5,6), it was considered important to examine the antitumor properties of the compound

[1]Supported by AID contract CSD 2837; by contract N01-CB-43967 from the Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare; by ICI United States, Inc; and by grants from the Yorkshire Cancer Research Campaign and ICI Ltd (Pharmaceuticals Division) in England.

[2]Department of Pharmacology, Leeds University School of Medicine, England.

[3]I would like to thank Suzanne Koerner and Carol Roberson for their assistance in the studies undertaken at the Worcester Foundation for Experimental Biology, Shrewsbury, Mass, and Margaret M. Collins, C. J. Dix, Lynne J. Dowse, T. Jaspan, G. Prestwich, Linda Rowsby, and P. A. Rylett at Leeds University, England, for their invaluable assistance.

*Reprint requests to: V. Craig Jordan, PhD, Department of Pharmacology, Leeds University School of Medicine, Leeds LS2 9NL, England.

in the rat; if the mechanism of action were fully understood then a more rational approach to the treatment of man might be undertaken. The 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced rat mammary carcinoma model was selected since, as in man, there appear to be varying degrees of hormone dependence (7,8) with a spectrum of responses to antihormone therapies (9–11). In the second section of this paper the antitumor effects of tamoxifen will be considered and a mechanism of action in vivo will be proposed.

Antiestrogenic Properties of Tamoxifen in the Rat

In the earlier experiments of Harper and Walpole (2), tamoxifen was tested for estrogenic and antiestrogenic effects on immature rat uteri after oral administration. To exclude the possibility that the pharmacologic properties of tamoxifen were different after administration by the more convenient sc route, the estrogenic and antiestrogenic properties were re-examined. As previously noted, tamoxifen exhibits the properties of a partial agonist and even at large daily doses it can produce increases in uterine wet weight which are only approximately half of those attained with estradiol (fig 2). In antiestrogenic tests tamoxifen is active in the range of partial agonistic response, ie, tamoxifen only exhibits antagonistic properties at those doses that when administered alone will produce significant increases in uterine weight. To understand the mechanism of action of tamoxifen it was important to determine whether its effects were reversible or irreversible. As seen in figure 3, increasing daily doses of estradiol can produce increases in uterine weight in animals receiving concomitant tamoxifen administrations; thus the effects of the antiestrogen appear to be reversible. A similar conclusion has been reached in studies of ^{3}H-thymidine incorporation into human breast cancer cells in culture (12). Tamoxifen will inhibit estrogen-stimulated incorporation of ^{3}H-thymidine into DNA but the blockade can be reversed by further additions of estradiol.

In estrogen target tissues (uterus, vagina, pituitary, hypothalamus, or breast adenocarcinomata), estradiol is believed to bind to cytoplasmic estrogen-binding proteins (receptors) which are then transformed to a protein which has high affinity for the cell nucleus (13). After translocation to the nucleus there is an increase in RNA synthesis (14) followed by an increase in cytoplasmic protein synthesis (15) which also appears to result in a replenishment of cytoplasmic estrogen receptors (16). DNA synthesis and cell division occur approximately 24 hours after the first exposure to estradiol.

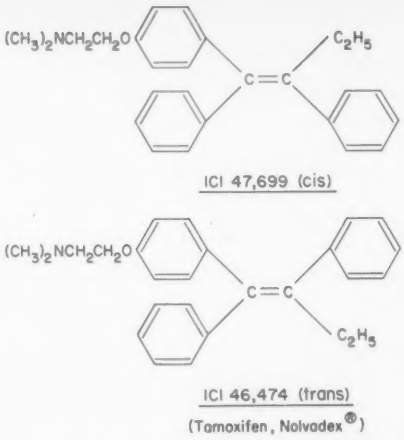

FIGURE 1.—Structure of tamoxifen and its *cis* isomer.

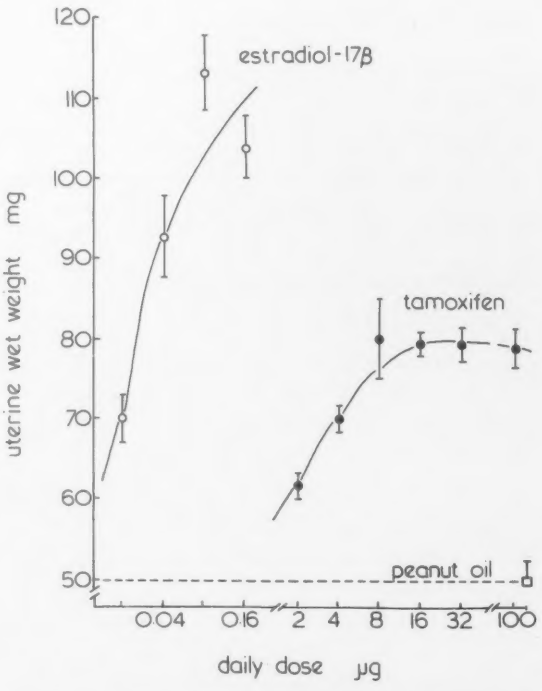

FIGURE 2.—Estrogenic activity of estradiol-17β or tamoxifen in the immature rat uterine weight test. Rats were randomized into groups (8 rats/group) and injected (sc) with varying doses of either estradiol or tamoxifen in 0.1 ml peanut oil on 3 consecutive days. Control animals were injected with peanut oil alone. On the 4th day animals were killed and the uteri were dissected out and weighed wet. Results are represented as mean ± SEM for groups.

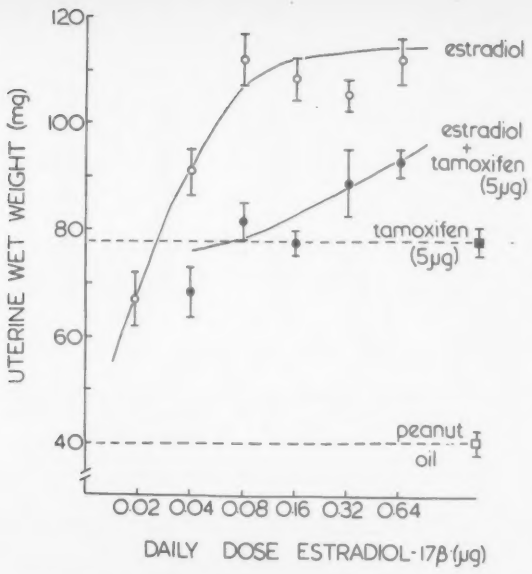

FIGURE 3.—Antiestrogenic effect of tamoxifen in the immature rat uterine weight test. Rats were randomized into groups (8 rats/group) and injected (sc) daily for 3 days with varying doses of estradiol-17β, tamoxifen (5 μg), or varying doses of estradiol and tamoxifen (5 μg). All injections were given in 0.1 ml peanut oil, with multiple injections made at separate sites. Animals were killed on the 4th day and the uteri were dissected out and weighed wet. Results are represented as mean ± SEM for groups.

The mechanism of action of antiestrogens may result from an interference with the estrogenic stimulus at a variety of stages. Potentially, nonsteroidal antiestrogens may inhibit the receptor binding of estradiol, impair transformation, alter the translocation process, or inhibit the synthesis of new cytoplasmic estrogen receptors thereby denying the cell nucleus a sustained flow of estradiol-estrogen receptor complexes.

Tamoxifen exhibits the properties of a competitive antagonist in vitro (17) since it will reversibly inhibit estradiol binding to rat uterine estrogen receptors. Furthermore, using the technique of sucrose density gradient analysis, tamoxifen will completely inhibit the binding of ^{3}H-estradiol to rat uterine estrogen receptors which sediment in the 8S region of the gradient (fig 4). Similar effects are observed with cytosols derived from human proliferative endometrium (18) and human breast and uterine adenocarcinomata (19).

After the antiestrogen binds to the estrogen receptor in the cytoplasm, it is not known whether the complex transforms in a manner analogous to the estradiol-estrogen receptor complex that is converted from a 4S to a 5S unit (20). Whether transformation is completed or not, the translocation process to the nucleus appears to occur. Exchange assay techniques, used to determine filled nuclear sites (21), have revealed that antiestrogens, notably nafoxidine (22,23), will enter the nucleus in a way similar to estradiol. Similarly, we have used the same technique to show that tamoxifen will facilitate the transfer of estrogen-receptor complexes to the rat uterine cell nucleus (unpublished observation). Therefore, an impaired transfer of receptor complex to the nucleus does not appear to be an antiestrogenic mechanism.

The replenishment of uterine cytoplasmic estrogen receptors has been suggested to be the primary mechanism of action of the nonsteroidal antiestrogens (24). The theory is based upon the observation that whereas estradiol and nonsteroidal antiestrogens produce a rapid depletion of cytoplasmic estrogen receptors, with the units passing to the cell nucleus, only estradiol-treated animals replenish the cytoplasmic receptors by 24 hours. Antiestrogen-treated animals have a prolonged depletion of cytoplasmic estrogen receptors for several days. This effect is illustrated in figure 5. Ovariectomized rats were treated with estradiol (1 μg) or tamoxifen (200 μg) and at 8 hours the estradiol-treated animals had depleted cytoplasmic receptors but at 24 hours an excess of estrogen receptors was present. By comparison, the tamoxifen-treated animals had low levels of cytoplasmic estrogen receptors at 24 hours. After multiple administrations this pattern continued with estradiol-treated groups replenishing cytoplasmic receptors within 24 hours, whereas tamoxifen-treated animals maintained a low level of cytoplasmic estrogen receptors suggesting that "receptor resynthesis" was not occurring.

One of the major pharmacokinetic differences between the administration of estradiol and tamoxifen is that whereas blood levels of estradiol are maintained for only a few hours (26), the biologic half-life of tamoxifen is several days (27). The possibility therefore exists that the level of binding ligand in the blood is the important factor in determining cytoplasmic estrogen receptors, ie, low levels of binding ligands allow cytoplasmic receptors to accumulate while high levels cause an equilibrium shift of cytoplasmic receptors to the nucleus thus producing an apparent long-term retention. If this concept is true then suitable doses of estrogens and antiestrogens would be equally effective in depleting cytoplasmic receptors for prolonged periods. To examine this theory we used three test systems: the effects of the estrogenic *cis* isomer of tamoxifen in the ovariectomized rat, the effects of tamoxifen in the mouse, and the effect of the long-acting estrogen,

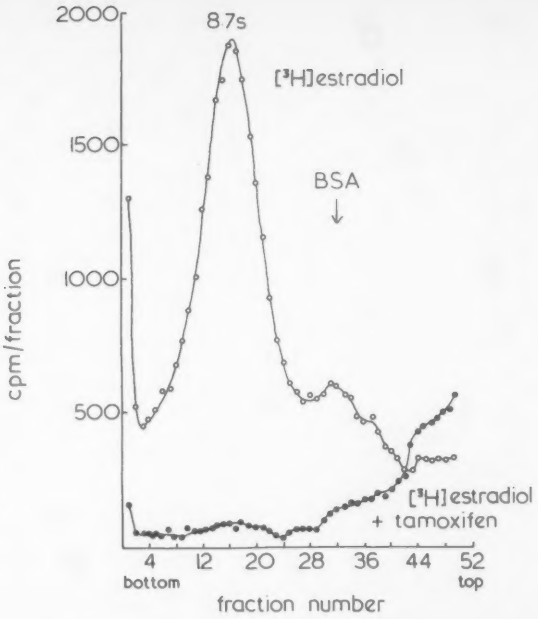

FIGURE 4.—Effect of tamoxifen (1×10^{-6} M) on the binding of 2,4,6,7-³H-estradiol (2×10^{-9} M) in rat uterine cytosol. Rat uteri were homogenized (4°C) in 4 vols of 0.01 M Tris-EDTA (1.5 mM) buffer containing 0.5 mM dithiothreitol (TED buffer) (pH 7.4). The 100,000 × g_{av} supernatant (cytosol) was incubated (4°C) with either ³H-estradiol or ³H-estradiol and tamoxifen for 4 hrs. Incubates were pipetted onto charcoal pellets to absorb unbound estradiol (30 mins at 4°C) and then 200-μl samples were layered onto 5%–20% sucrose gradients in TED buffer. Samples were centrifuged for 15 hrs (4°C) at 200,000 g_{av} in a Griffin Christ Omega II Ultracentrifuge and 100-μl fractions were collected by needle puncture of tubes; samples were then counted as previously described (54). Bovine serum albumin (BSA) was run on a separate gradient as a sedimentation standard (4.6S).

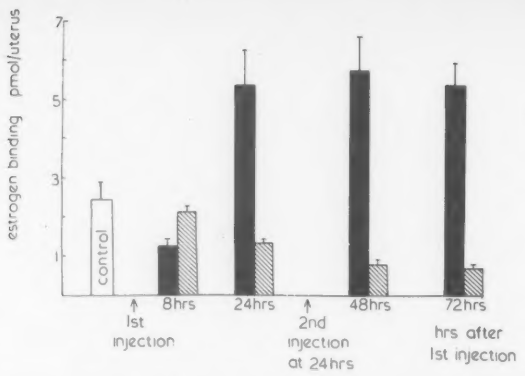

FIGURE 5.—Levels of total cytoplasmic uterine estrogen receptors after the sc administration of estradiol (1 μg) or tamoxifen (200 μg) in 0.1 ml peanut oil to ovariectomized rats. Cytosol was prepared as before (fig 4) and the exchange assay was essentially that described by Katzenellenbogen and co-workers (25). Administrations were made to groups of rats (5 rats/group) at 0, 24, and 48 hrs and assays were performed at 8, 24, 48, and 72 hrs. Black columns = estradiol-treated groups; shaded columns = tamoxifen-treated groups.

estradiol benzoate, in the immature rat. In each case there was a prolonged depletion of cytoplasmic estrogen receptors but a stimulation of DNA synthesis as an expression of estrogen effectiveness. Therefore, the inability to measure cytoplasmic estrogen receptors is not an exclusive property of antiestrogenic activity. Since it has been shown that estrogen-receptor reaccumulation may be a function of the dose of the antiestrogen (28), we propose that receptor resynthesis may occur at a low level to maintain a total cellular concentration, but the new cytoplasmic receptor never accumulates in the presence of high concentrations of binding ligand because the receptor units are translocated to the nucleus.

If antiestrogens and estrogens both cause a transfer of receptors to the nucleus, the quality of the units rather than the quantity must be the important factor involved in the initiation of cell division. As seen in table 1, tamoxifen is unable to provoke sustained DNA synthesis in the ovariectomized rat uterus although uterine wet weight and uterine protein content (unpublished observation) increases. If the occupation of a few nuclear acceptor sites by estradiol-estrogen receptor complexes (30) is necessary for true uterine growth, then occupation by estrogen receptors bound to tamoxifen may be unable to initiate the events leading to cell division. Since antiestrogens are able to activate RNA polymerase (31) and ultimately stimulate protein synthesis, then the nuclear events necessary for this process must be less specific than the stimulation of DNA synthesis.

In conclusion therefore, tamoxifen may act as an antiestrogen by competing for the cytoplasmic estrogen receptor and then tamoxifen-estrogen receptor complexes may undergo an imperfect transformation process. Whether transformation occurs or not, both estradiol-estrogen receptor complexes (true messenger unit) and tamoxifen-estrogen receptor complexes (false messenger unit) will be translocated to the nucleus and true and false messenger units will compete for the few nuclear acceptor sites which specifically stimulate DNA synthesis and cell division (fig 6). Therefore, the relative concentrations of estrogen and antiestrogens in the immediate area of the target tissue would ultimately determine estrogenic or antiestrogenic effects on uterine growth.

TABLE 1.—Uterine wet weights and prepared nuclear DNA content 24 hrs after 1, 2, or 3 administrations of estradiol (1 μg) or tamoxifen (200 μg) in 0.1 ml of peanut oil at 24-hr intervals to ovariectomized Sprague-Dawley rats*

	Uterine wet wt (mg ± SEM) (Nos. of administrations)			DNA content (μg ± SEM) (Nos. of administrations)		
Treatment	1	2	3	1	2	3
Estradiol	265±25†	277±9†	332±20†	236±19‡	324±19†	355±19†
Tamoxifen	212±7‡	207±7‡	198±8‡	204±13§	213±8§	208±24§
Control		125±5			178±16	

*Upon sacrifice uteri were excised, cleared of adhering fat, and homogenized individually in 1 ml of Tris (0.01 M)-EDTA (1.5 mM) buffer (pH 7.4) by 3 × 10 sec burst of an Ultraturrax tissue homogenizer. The homogenate was centrifuged at 150 × g_{av} for 5 mins to separate unhomogenized cells and muscular tissue. The supernatant was pipetted off and stored, and the process was repeated with the precipitate resuspended in buffer. The pooled supernatants were centrifuged at 800 × g (4°C) for 10 mins to prepare the nuclear pellet and then this was resuspended in buffer containing 0.25 M sucrose and recentrifuged at 800 × g (4°C) for 10 mins. DNA determinations were undertaken using the methods described by Burton (29). Treatment groups = 5 rats/group; control group = 18 rats. P values determined by student's t test (vs control).

†$P < 0.01$.

‡$P < 0.05$.

§$P > 0.05$.

Antitumor Properties of Tamoxifen in the Rat

The DMBA-induced rat mammary carcinoma was first described by Huggins and co-workers (7) as a potential model for the study of hormone-dependent cancer. Tumor production is relatively simple since administration of DMBA to 50-day-old female Sprague-Dawley rats results in the appearance of multiple adenocarcinomata 60–200 days later. A stable hormone environment is necessary for tumor induction since ovariectomy (32) or antihormone therapy (33–35) inhibits or retards the production of tumors. Similarly, a dose of tamoxifen which produces a long-term antiestrogenic effect will inhibit the induction of mammary carcinomata if administered with the DMBA (36,37).

Established DMBA-induced rat mammary carcinomata respond to ovariectomy, hypophysectomy (38), and antihormone therapies (9–11), therefore these tumors were considered to be a suitable model to study the mechanism of action of tamoxifen as an antitumor agent.

The daily administration of tamoxifen inhibits the growth of established DMBA-induced tumors (fig 7) although this effect appears to be reversible since once the antiestrogenic effects disappear, ie, when the compound is cleared from the body, some of the tumors resume growth. Tumor 6 for example (fig 7) rapidly regressed during tamoxifen therapy but started to regrow when therapy was stopped. In further experiments not shown in the figure this tumor regressed upon ovariectomy but regrew after the administration of estrogen, therefore this particular tumor appeared to be hormone dependent.

Each tumor responds individually to tamoxifen therapy with about 10%–15% of the tumor population completely unresponsive. We have studied the change in area of approximately 300 tumors during tamoxifen therapy and several types of response have been observed. The effects are illustrated in the hypothetical experiment shown in figure 8. Some tumors remain static whereas others (approximately 25%) disappear completely. Some tumors regress but then the therapy becomes ineffective and tumor growth resumes. By comparison, some tumors grow but after a few weeks the effects of tamoxifen therapy causes tumor regression. Therefore, the tumor population is completely heterogeneous and provides an excellent model for determining the unique mechanism of action of tamoxifen in the responsive tumors.

Mammary carcinomata induced by DMBA grow in response to estradiol (39) or prolactin (40) but since estradiol can stimulate prolactin release (41, and table 2) the precise mechanism for the hormonal control of tumor growth remains obscure. In fact, the possibility that both prolactin and estradiol are necessary for effective tumor homeostasis has been suggested (42). The complex hormonal interplay in the intact rat provides several

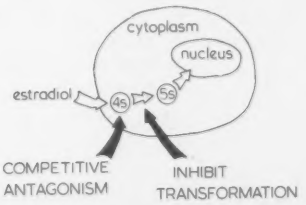

FIGURE 6.—Proposed model for the mechanism of action of tamoxifen in estrogen target tissues.

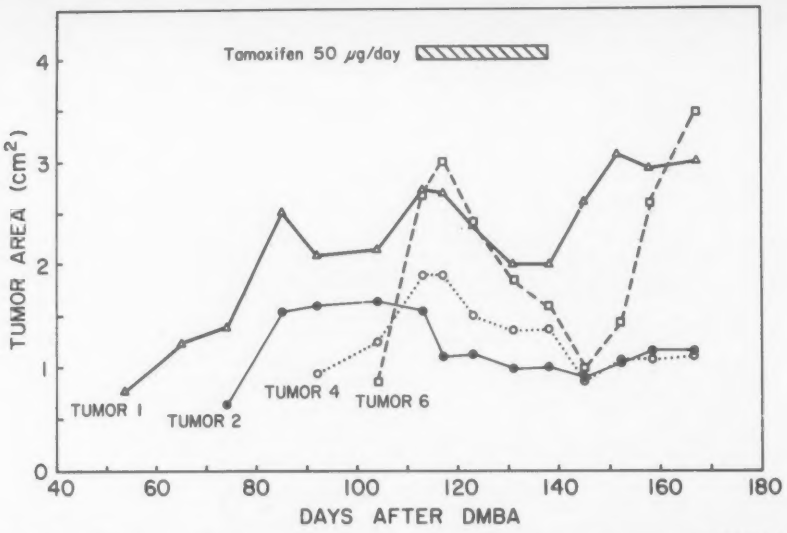

FIGURE 7.—Effect of daily sc administrations of tamoxifen (50 μg in 0.1 peanut oil) on the growth of DMBA-induced mammary carcinomata on a single female rat. Tumors were measured weekly with calipers in 2 dimensions: length (l), width (w), and tumor areas were calculated using the formula $\pi \times \frac{l}{2} \times \frac{w}{2}$. Estrous cycles stopped during tamoxifen therapy and restarted 1 wk after the cessation of treatment.

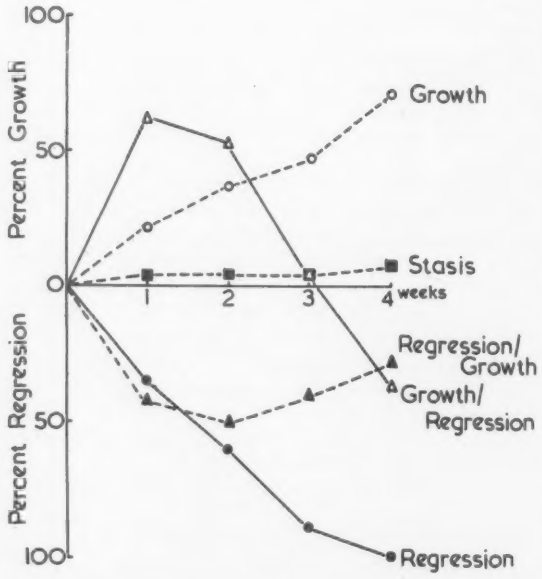

FIGURE 8.—Types of DMBA-induced carcinoma growth or regression responses observed during continuous tamoxifen therapy. Tumor area is represented as a percentage increase or decrease of the untreated tumor area.

potential mechanisms of action for tamoxifen and these are illustrated in figure 9. We have considered that tamoxifen may inhibit estradiol-stimulated prolactin release thereby causing prolactin-dependent tumors to undergo regression. Alternatively, estrogen-dependent tumors may regress if tamoxifen impairs the ovarian production of estradiol or blocks the effects of estradiol at the tumor level. Each of these potential mechanisms will be considered in turn in order to formulate a possible mechanism of action for tamoxifen.

Nonsteroidal antiestrogens partially inhibit estrogen-stimulated rises in plasma prolactin (33,43), therefore decreases in circulating prolactin may produce regression due to a decrease in activation of the prolactin receptor (44). Tamoxifen will produce a dose-related decrease in the rise in estradiol-stimulated uterine weights in the ovariectomized rat, but this trend is not seen in the circulating levels of prolactin (table 2). Tamoxifen will only produce about a 50% reduction in the circulating prolactin levels and is unable to reduce plasma levels to those found in the ovariectomized rat. Similarly, in the intact rat there is no correlation between the circulating levels of prolactin and tumor response to tamoxifen therapy (45). Therefore reduction of circulating prolactin levels as an antiestrogenic action of tamoxifen would appear not to be a primary antitumor effect.

TABLE 2.—Mean uterine wet weights and plasma prolactin levels (± SEM) of ovariectomized rats treated for 8 days with sc injections of estradiol alone or in combination with increasing doses of tamoxifen*

Tamoxifen (μg/day)	Estradiol (μg/day)	Uterine wet wt (mg)	Plasma prolactin levels (ng/ml)
—	—	152±7†	22.8±5.6†
—	5	515±19	304.4±48.4
12.5	5	443±19§	204.8±27.3‡
50.0	5	378±27†	179.4±16.8¶
200.0	5	290±11†	183.1±4.7¶
800.0	5	252±13†	172.9±27.9¶

*Treatment groups = 8 rats/group. For further details of the methods, *see* ref 45. *P* values determined by comparison with estradiol-treated rats (Student's t test).
†$P < 0.001$.
‡$P > 0.05$.
§$P < 0.02$.
¶$P < 0.05$.

Tamoxifen has been found to reduce circulating levels of estradiol in the rat (46) by inhibiting the synthesis of estradiol in the ovary (47), therefore this type of chemical oophorectomy may provide a mechanism for antitumor activity or at least facilitate the competitive blockade by tamoxifen of estradiol's effects at the tumor level (48,49). To determine whether tamoxifen would still provoke tumor regression in the presence of constant estradiol therapy, rats with DMBA-induced tumors were ovariectomized and treated with either estradiol (5 μg/day) or estradiol (5 μg/day) and tamoxifen (200 μg/day). After 4 weeks of treatment estradiol-treated animals had a mean increase in tumor area (from 2.5 ± 0.6 cm^2 to 4.8 ± 1.4 cm^2, five tumors) whereas animals receiving tamoxifen and estradiol had undergone a mean tumor regression (from 1.7 ± 0.4 cm^2 to 0.6 ± 0.5 cm^2, six tumors; at 4 weeks $P < 0.05$ by student's t test).

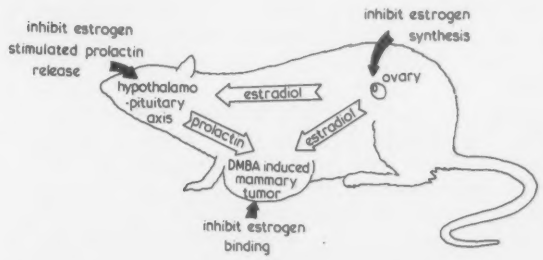

FIGURE 9.—Hormonal control of DMBA-induced mammary tumor growth and the potential methods by which tamoxifen might precipitate tumor regression.

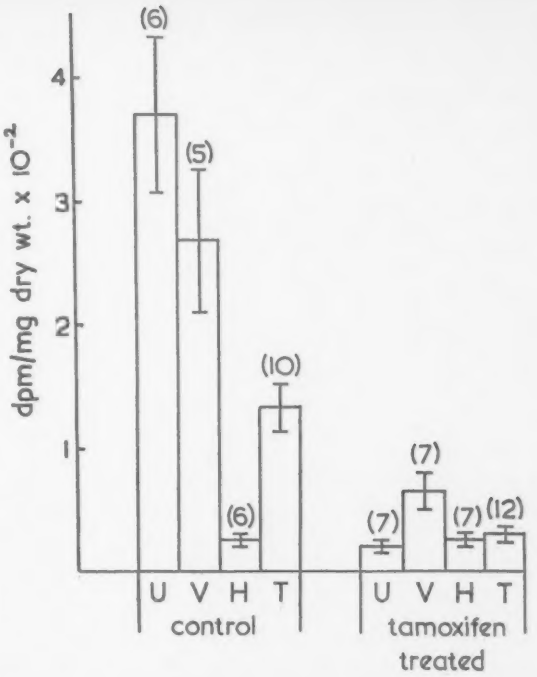

FIGURE 10.—Binding of 6,7-^{3}H-estradiol (30 microcuries) in vivo in rat uterine (U), vaginal (V), heart (H), and DMBA-induced tumor (T) samples. Animals were treated with either peanut oil (control) or tamoxifen (200 μg/day sc) for 3 wks to provoke significant mean tumor regression ($P < 0.05$). Animals were killed 4 hrs after ^{3}H-estradiol administration and tissues were processed using the methods described in ref 54. Nos. of samples are shown in parentheses. Comparison by student's t test between treated and control rats: U = $P < 0.001$; V = $P < 0.01$; T = $P < 0.001$; H = $P > 0.05$.

Therefore it would appear that tamoxifen is an effective antitumor agent in the presence of estradiol and although impaired estrogen synthesis may occur in the intact rat, a reduction in circulating estradiol is not essential for antitumor effects.

Tumors induced by DMBA administration have been reported to bind estradiol and have an estrogen-receptor mechanism similar to that found in the rat uterus (50–53). Tamoxifen, which usually produces a regression of DMBA-induced tumors, also inhibits the binding of ^{3}H-estradiol to tumor tissues in vivo (54). In figure 10 two groups of rats with DMBA-induced tumors were treated with either peanut oil (control) or tamoxifen (200 μg/day) for 3 weeks to provoke tumor regression. Animals were then injected with ^{3}H-estradiol, but whereas control animals bound the radioactivity in the target tissues (uterus, vagina, and DMBA-induced tumors),

in tamoxifen-treated animals the binding of estradiol was impaired.

The estrogen-binding protein of DMBA-induced tumors sediments in the 8S region of sucrose density gradients (39,53) and tamoxifen effectively inhibits the binding of ^{3}H-estradiol to this protein (54). Therefore tamoxifen may exert an antiestrogenic effect in the tumor cells in a manner similar to that previously discussed for the rat uterus.

Jensen and co-workers (55) have suggested that the presence of the estrogen receptor in human breast cancer may indicate the dependence of the tumor upon estrogens. Since DMBA-induced tumors exhibit a spectrum of estrogen-binding activities (56), we have applied Jensen's concept to experimental rat mammary cancer in an attempt to rationalize the heterogeneous responses to tamoxifen. Rats with established DMBA-induced tumors (approximately 2 cm^2 cross-sectional area) were biopsied and the estrogen-receptor concentration was determined using a sensitive sephadex LH20 method (57) for separating bound and free ^{3}H-estradiol after incubations (30°C for 30 minutes). To estimate estrogen binding a single concentration of ^{3}H-estradiol was used and specific binding (fmol/mg cytosol protein) was plotted against the response of the tumor (percentage increase or regression) after 3 weeks of tamoxifen therapy (50 μg/day) (fig 11). The majority of tumors were biopsied at diestrus, a time of low levels of circulating estrogen (58), although some were biopsied at estrus or proestrus to determine whether false positive or false negative results would occur. No major alteration in the general trend appeared to occur suggesting that with this method, the time of the estrous cycle during which biopsies were taken was not critically important (59). However, dramatic decreases in circulating estrogen levels appear to enhance estrogen-receptor measurement, for example, 24 hours after ovariectomy DMBA-induced tumors have a pronounced increase in the levels of estrogen receptors (60) and, similarly, in human breast cancer postmenopausal levels of estrogen receptors tend to be higher than premenopausal levels (61). Furthermore, in the premenopausal patient with high levels of circulating estradiol there are lower estrogen-receptor concentrations in the breast tumor (62).

Three major types of tumor response to tamoxifen therapy occur in the rat (63) (fig 12). At low levels of estrogen binding (group A) the tumors either remain static or grow during therapy. As the concentration of estrogen receptors in the biopsy samples increases (group B) there is a greater probability of increased tumor regression in response to tamoxifen. At high concentrations of estrogen receptors (group C) the mean tumor response to therapy is

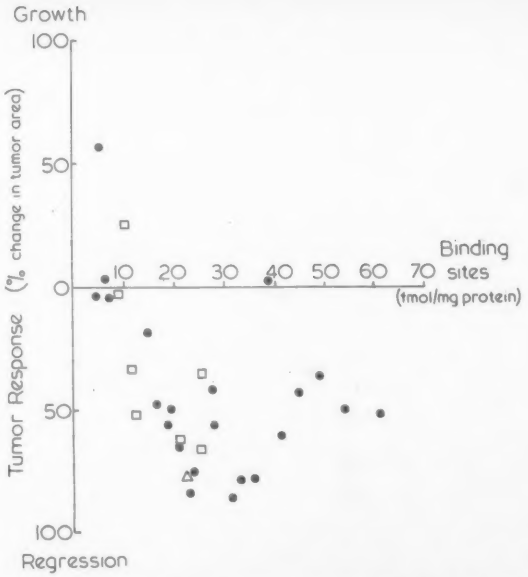

FIGURE 11.—Graph of the percentage change in DMBA-induced tumor area after 3 wks of tamoxifen therapy (50 μg/day) plotted against the cytosol concentration of estrogen-binding sites derived from biopsy samples taken before therapy. For detailed methods, see ref 59. Times of the estrous cycle when biopsies were taken: circles, diestrus; squares, estrus; triangle, proestrus.

only about 50% regression instead of the total tumor regression that might have been expected if all the tumor cells were estrogen dependent. The possibility exists that a larger dose of tamoxifen could have provoked further tumor regression, but since estrogen-receptor binding in the tumors is reduced at this dose level (50 μg/day) (59) this appears to be unlikely. One explanation for the partial response of group C is that false positive determinations have occurred since there may have been a high density of cells containing estrogen receptors in the region of the biopsy. Alternatively, these tumors contain a few cells with abnormally high levels of estrogen receptors, therefore the partial tumor regression is a result of the control of the few receptor-containing cells.

Although tamoxifen inhibits estrogen binding in the DMBA-induced tumor cells, other circulating hormones, notably prolactin, may be able to circumvent the antiestrogen block and stimulate cell growth by an alternative route. Certainly tamoxifen is unable to effectively control circulating levels of prolactin (45) and this may activate prolactin receptors which have been found in tumors with high levels of estrogen receptors (64). In this context it is interesting to note that nafoxidine, a structurally

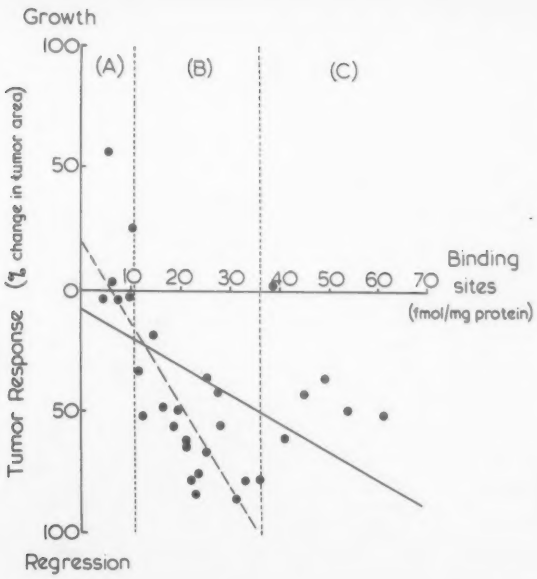

FIGURE 12.—Graph of the types of DMBA-induced tumor response to 3 wks of tamoxifen therapy (50 μg/day) based upon the concentration of estrogen receptors in biopsy samples taken prior to therapy. For detailed methods, see ref 63. For areas A and B, $P < 0.001$ for linear regression. For areas A, B, and C, $P < 0.01$ for linear regression.

related antiestrogen, is unable to control the growth of DMBA-induced tumors which are dependent upon prolactin (65).

In conclusion, the potential mechanisms of action of tamoxifen as an antitumor agent in the rat have been surveyed. From the data presented it would appear that tamoxifen exerts an antitumor effect by blocking estrogen receptors in the tumor cells (fig 13). Tumors with low levels of estrogen receptors, which are perhaps estrogen independent, do not undergo regression during tamoxifen therapy whereas tumors containing estrogen receptors regress. Tamoxifen may therefore exert an antiestrogenic effect in the tumor cell in a way similar to that proposed for the uterine cell.

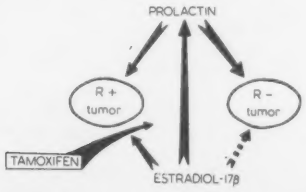

FIGURE 13.—Proposed mechanism of action of tamoxifen to provoke tumor regression in the DMBA-induced rat mammary carcinoma model.

The objective of the antitumor studies was an attempt to formulate a mechanism of action for tamoxifen in a laboratory model for breast cancer so that any conclusions could be applied towards the treatment of the human condition. Since there are a spectrum of estrogen-receptor concentrations in human breast carcinomata (66), and tamoxifen inhibits the binding of ^{3}H-estradiol in vitro to estrogen-receptor proteins derived from human breast tumors (19,67), only those tumors with estrogen receptors may be sensitive to tamoxifen therapy. An estrogen-receptor assay before therapy may therefore exclude unresponsive, estrogen-receptor negative patients and redirect their treatment along a more productive route (68).

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Interactions of Antiestrogens With Human Breast Cancer in Long-Term Tissue Culture

Marc Lippman,* Gail Bolan, and Karen Huff[1,2]

SUMMARY

A variety of antiestrogens can be shown to antagonize estrogen action in animal model systems. Several of these compounds are useful in the management of metastatic human breast cancer. To further elucidate their mechanism of action, we studied several of these compounds using human breast cancer cell lines maintained in long-term tissue culture as a model system. Antiestrogens including tamoxifen (NSC-180973; ICI-46474), nafoxidine, CI-628, and clomiphene citrate inhibit macromolecular synthesis below control levels in two human breast cell lines. This effect is limited to cell lines which contain estrogen receptors. Simultaneous addition of as little as 1000-fold less estradiol prevents antiestrogen effects. Sequential addition of estrogen for up to 48 hours to cells incubated in antiestrogen reverses inhibition. If cells are continued in antiestrogen alone for more than about 3 days, inhibitory effects become irreversible. The cells detach from the surface of the culture vessel and are no longer viable. Tamoxifen competes with ^{3}H-estradiol for specific receptor sites but with about a 100-fold lower apparent affinity. Direct binding of ^{3}H-tamoxifen and ^{3}H-estradiol to duplicate cytoplasmic extracts reveals equivalent numbers of binding sites but a 20-fold lower affinity for the antiestrogen. There is reasonable agreement between concentrations of tamoxifen which bind to receptor and concentrations which inhibit cells.

[Cancer Treat Rep 60:1421–1429, 1976]

Breast cancer is frequently estrogen responsive. Dimethylbenz(*a*)anthracene (DMBA)-induced rat mammary cancer regresses about 80%–90% of the time following castration and may be restimulated by subsequent estrogen administration (1,2). Approximately one of three premenopausal patients with metastatic breast cancer will have objective tumor regressions following castration (3). Paradoxically perhaps, an equal proportion of postmenopausal women with disseminated cancer will have objective tumor responses when treated with pharmacologic concentrations of estrogen (4). In both groups of patients, the presence of estrogen receptor in tumor samples is associated with an increased response rate, while the absence of receptor has suggested that objective responses would be seen $< 10\%$ of the time (5). Recent studies have shown that antiestrogens can antagonize the uterotropic effects of estrogen (6), lead to regressions of DMBA-induced rat mammary carcinomas (7), and induce objective clinical responses in about one of three postmenopausal patients with metastatic breast cancer (8). While these compounds can compete with estrogen for specific cytoplasmic steroid receptor sites (9), recent work in rodents by Clark et al (10) and Katzenellenbogen et al (11) has suggested that their mechanism of action may be more complex. They have provided data suggesting that antiestrogen can

[1]Medicine Branch, Clinical Oncology Program (COP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

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*_Reprint requests to:_ Marc Lippman, MD, Medicine Branch, COP, DCT, NCI, Bldg 10, Rm 6B04, National Institutes of Health, Bethesda, Md 20014.

bind to estrogen receptor and these complexes can translocate to nuclear sites. Nuclear occupancy time and salt extractability of (antiestrogen-estrogen receptor) complexes are different from those of nuclear-associated estrogen-receptor complexes.

While studies in animals and man have shed much light on the mechanisms by which estrogens induce trophic changes in target tissues, these complex systems have disadvantages. First, it may be difficult to separate direct effects of hormones on target tissues from effects mediated by alterations in levels of activity of other trophic hormones. For example, glucocorticoids are required to observe optimal activity of glucagon or catecholamines. Thus, glucocortiocides are "permissive" for the effects of other hormones. Alternatively, in vivo, estrogens have obvious effects on gonadotropins, prolactin, growth hormone insulin, and many other hormones. Second, effects may not be due to a direct interaction of administered hormone with target tissues. The hormone may affect supporting stroma or the interaction of the tumor with surrounding cells, including the immune system. For these reasons, we felt that it would be of interest to examine the effects of estrogen and antiestrogens on human breast cancer cell lines maintained in tissue culture. Using defined, serum-free conditions, it should be possible to examine the effects of estrogen and antiestrogen in a more controlled way. We have previously shown that two of these cell lines in tissue culture contain estrogen receptor and respond to physiologic concentrations of estrogen (12–15). Antiestrogens inhibit macromolecular synthesis in these cells. In the present study we report additional experiments further characterizing the interactions of antiestrogens with these cell lines.

METHODS

Cells.—MCF-7 is a human breast cancer cell line established from a malignant pleural effusion in a postmenopausal woman with metastatic breast cancer (16). ZR-75-1 was also established from a malignant effusion in a postmenopausal woman with breast cancer. Both cell lines have been shown to be free of mycoplasma contamination (Litton Bionetics, Rockville, Md). The human and mammary nature of these cells has been discussed previously (12–15). Cells were grown in Improved Minimal Essential Medium (IMEM) supplemented with glutamine (0.6 g/liter) and antibiotics (NIH Media Unit), supplemented with 5% fetal calf serum (North American Biological, Miami, Fla). Cells were grown in monolayers in a humidified 5% CO_2 incubator maintained at 37°C.

Steroid receptor analyses.—Cells growing in IMEM plus 5% fetal calf serum were switched to serum-free IMEM. Twenty-four hours later, cells were harvested by washing the dishes once in ice-cold phosphate-buffered saline (PBS), pH 7.4, and then either suspending the cells with EDTA or scraping with a rubber policeman. Cells were collected by centrifugation at 800 × *g* for 5 minutes. Cell pellets were suspended in two or three volumes of TED buffer (10 mm Tris-HCl, pH 7.4; 1.5 mM EDTA; 0.5 mM EDTA; 0.5 mM dithiothreitol) and homogenized with 30 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 104,000 × *g* for 1 hour at 4°C. The supernatant cytosol was assayed for cytoplasmic receptor activity using minor modifications (17) of a previously published dextran-coated charcoal assay (18). Binding data were prepared for Scatchard analysis by computer-assisted methods (19). ^{3}H-estradiol (specific activity, 100 curies/mM, Amersham Searle, Arlington Heights, Ill) was used without further purification. The specific activity of ^{3}H-tamoxifen was 19.5 curies/mM.

Precursor incorporation studies.—Cells growing in IMEM plus 5% fetal calf serum were washed once with PBS and harvested with sterile trypsin-EDTA solution. The cell suspension was diluted (greater than tenfold) into fresh serum containing IMEM and replicately plated into plastic multiwell dishes. After the cells had attached to the surface of the dish the medium was removed by suction and replaced with serum-free IMEM. Eight hours later, this medium was removed and fresh serum-free IMEM was added. Twenty-four hours later, steroid hormones were added as 1000-times concentrates in ethanol. This concentration of ethanol (0.1%) has no effect on macromolecular synthesis. Twenty-four hours later, radiolabeled precursors were added. ^{3}H-thymidine (40 curies/mM) at a concentration of 0.05 microcurie/ml or ^{14}C-leucine (specific activity, 230 millicuries/mM) at a final concentration of 0.1 microcurie/ml was employed. Following a 1-hour labeling period, the cells were washed while attached to the dish surface with PBS and then suspended with EDTA solution. Cell pellets were collected by centrifugation for 1 minute in a serofuge (Clay Adams, Parsippany, NJ) and stored frozen until assayed for precursor incorporation (12) or protein content (20).

RESULTS AND DISCUSSION

The effects of estradiol and tamoxifen alone and in combination on MCF-7 human breast cancer are shown in figure 1. Using serum-free conditions, 10^{-9} M estradiol stimulates thymidine incorporation by 105%. Estradiol, at 10^{-8} M, does not further stimu-

TABLE 1.—Effects of 17β-estradiol and tamoxifen on thymidine incorporation in ZR-75-1 human breast cancer

Addition	Concentration (M)	Incorporation*
—	—	6.8 ± 0.7
17β-Estradiol	10^{-7}	12.9 ± 1.6
Tamoxifen	10^{-6}	3.9 ± 0.5
17β-Estradiol + Tamoxifen	10^{-7} + 10^{-6}	12.0 ± 1.1

*Results are expressed in dpm incorporated × 10^{-3}/mg of protein/hr; means of triplicate determinations ± 1 SD.

late thymidine incorporation. We have previously shown this response to range between twofold and fivefold in various experiments (12–15). Tamoxifen, at a concentration of 10^{-7} M, inhibits thymidine incorporation minimally below control levels, whereas 10^{-6} M tamoxifen decreases thymidine incorporation to about 50% of control. As increasing concentrations of estradiol are added, along with 10^{-6} M tamoxifen, reversal of the antiestrogen effect is seen. As little as a 1000-fold less estradiol yields levels of thymidine incorporation.

The blockade of antiestrogen effect by simultaneous estrogen administration which we observe with tamoxifen in MCF-7 cells is a different result than that obtained by Clark and co-workers using a rodent system (21). They found that in rat uterus the antiestrogen nafoxidine effectively prevented estrogen action when both were administered simultaneously. We therefore examined the effects of tamoxifen on another human breast cell line, ZR-75-1 (table 1). As indicated, 17β-estradiol stimulates precursor incorporation while tamoxifen inhibits the cells below control values. Tamoxifen effects are blocked by the simultaneous addition of tenfold less estradiol, also shown in the table. Thus, at least one other estrogen-responsive human breast cancer cell line shows a similar response to antiestrogen.

The effects of three other antiestrogens, nafoxidine (The Upjohn Company, Kalamazoo, Mich), CI-628 (nitromifene citrate) (Parke, Davis and Co, Detroit, Mich), and clomiphene citrate (Merrell-National Laboratories, Cincinnati, Ohio) on MCF-7 cells are shown in tables 2–4.[3] Each of these antiestrogens inhibits macromolecular synthesis below control levels. In each case, simultaneous addition of 17β-estradiol completely or nearly completely

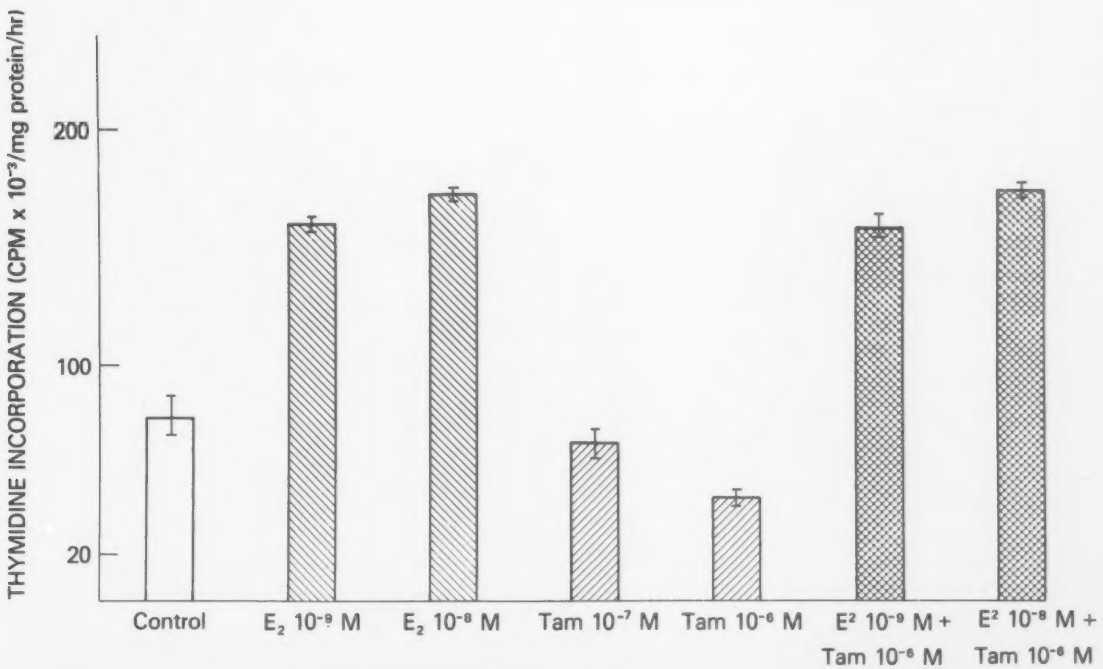

FIGURE 1.—Effects of 17β-estradiol and tamoxifen alone and in combination on MCF-7 human breast cancer. Results are means of triplicate determinations ± 1 SD. Techniques are supplied in the Methods section.

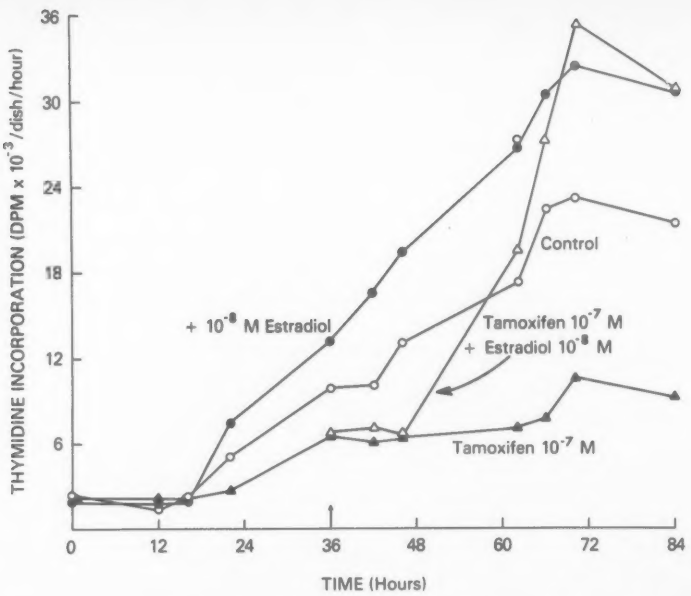

FIGURE 2.—Kinetics of the response of MCF-7 human breast cancer to either 17β-estradiol or tamoxifen. At 36 hrs (arrow), sufficient 10^{-5} M estradiol to yield a final concentration of 10^{-8} M was added to ½ of the dishes previously incubated with tamoxifen alone. Results are means of triplicate determinations; SDs are generally < 10%.

blocks this inhibitory effect. In each case, as a control, the stimulatory effects of 17β-estradiol alone are provided. Thus, four different antiestrogens all show roughly equivalent interactions with estradiol in a human breast cancer cell line.

If these observations apply to clinical breast cancer then one might predict that antiestrogens might be less efficacious in premenopausal women because higher concentrations of circulating estrogens might prevent antiestrogen effect. This may be an oversimplification. In vivo, antiestrogens such as tamoxifen may have a variety of actions including suppression of gonadotropins. Furthermore, elsewhere in the proceedings of this workshop may be found reports of objective clinical responses to tamoxifen in premenopausal women. Thus, some caution is indicated in concluding that antiestrogen effects observed in vitro parallel those seen in vivo.

The kinetics of estrogen and antiestrogen effects are shown in figure 2. Replicately plated cells are exposed to either tamoxifen (10^{-7} M) or 17β-estradiol (10^{-8} M). Nucleoside is added for 1 hour prior to harvesting the cells. As shown, for about the first 14 hours there are no differences between hormone-treated and control cells. Following this time, estradiol stimulates and antiestrogen inhibits thymidine incorporation. After 36 hours, one half of the dishes containing tamoxifen had a sufficient 17β-estradiol added to yield a final concentration of 10^{-8} M,

TABLE 2.—Effects of 17β-estradiol and nafoxidine on thymidine incorporation in MCF-7 human breast cancer

Addition	Concentration (M)	Incorporation*
—	—	75.8 ± 8.1
17β-Estradiol	10^{-9}	107.0 ± 5.3
	10^{-8}	106.0 ± 3.1
Nafoxidine	10^{-7}	70.2 ± 6.3
	10^{-6}	57.6 ± 0.8
Nafoxidine + 17β-Estradiol	10^{-7} + 10^{-9}	88.6 ± 2.1
Nafoxidine + 17β-Estradiol	10^{-7} + 10^{-8}	95.4 ± 6.1

*Results are expressed in dpm incorporated/mg of protein/hr; means of triplicate determinations ± 1 SD.

tenfold less than that of the antiestrogen already present. For an additional 12–14 hours there was no difference between cells incubated in tamoxifen alone and those incubated in tamoxifen plus estradiol. This was followed by a striking reversal of antiestrogen effect during which thymidine incorporation increased rapidly in dishes containing both estrogen and antiestrogen. The steeper rise in thymidine incorporation in tamoxifen plus estradiol-treated cells, as compared with the cells in estrogen alone, is consistent with some degree of cell syn-

TABLE 3.—Effects of 17β-estradiol and CI-628 on thymidine incorporation in MCF-7 human breast cancer

Addition	Concentration (M)	Incorporation*
—	—	17.0 ± 3.7
CI-628	10^{-7}	15.8 ± 2.7
CI-628	10^{-6}	8.7 ± 1.0
17β-Estradiol	10^{-8}	37.6 ± 5.0
17β-Estradiol + CI-628	10^{-8} + 10^{-7}	48.6 ± 14.0
17β-Estradiol + CI-628	10^{-8} + 10^{-6}	36.2 ± 11.0

*Results are expressed in dpm incorporated/mg of protein/hr; means of triplicate determinations ± 1 SD.

TABLE 4.—Effects of 17β-estradiol and clomiphene citrate on thymidine incorporation in MCF-7 human breast cancer

Addition	Concentration (M)	Incorporation*
—	—	78.6 ± 9.4
Clomiphene citrate	10^{-9}	90.8 ± 17.0
Clomiphene citrate	10^{-8}	95.6 ± 2.0
Clomiphene citrate	2×10^{-7}	59.9 ± 4.4
Clomiphene citrate	10^{-6}	48.0 ± 7.3
17β-Estradiol	10^{-9}	144.0 ± 5.0
17β-Estradiol	10^{-8}	132.0 ± 0.7
17β-Estradiol + Clomiphene citrate	10^{-9} + 10^{-7}	148.0 ± 5.0
17β-Estradiol + Clomiphene citrate	10^{-8} + 10^{-7}	152.0 ± 2.1

*Results are expressed in dpm incorporated/mg of protein/hr; means of triplicate determinations ± 1 SD.

chrony induced by the antiestrogen. We have observed this reversal phenomenon repeatedly, and the peak of thymidine incorporation rate seen in "rescued" cells usually exceeds that seen in cells treated with estrogen alone, further suggesting some degree of synchronous arrest induced by antiestrogen. If the cells are left in antiestrogen, after about 48–72 hours the cells begin to round up on the bottom of the culture vessel, detach, and die, although surviving cells are occasionally observed. Results shown in figure 2 are expressed on a per-replicately-plated-dish basis. Because estrogen stimulates cell growth and antiestrogens inhibit cell division (13), a slightly different result is obtained if the data are expressed on a per-mg-protein basis. Then, estradiol-induced increases in cell protein slightly diminish the fold of estrogen stimulation. Antiestrogen-induced slowing of cell division increases the apparent degree of inhibition of thymidine incorporation as compared with control. The importance of understanding the mechanism of estrogen and antiestrogen mediated effects on DNA synthesis is obvious. We have recently begun an investigation of hormonal control of enzymes involved in pyrimidine biosynthesis and phosphorylation. We have studied regulation by estrogen of thymidine kinase. Thymidine kinase catalyzes the formation of thymidylate from thymidine. We found good general agreement between enzyme activity and rates of thymidine incorporation both in estrogen-stimulated and tamoxifen-inhibited cells. However, in other studies (14,22) we have shown that induction of thymidine kinase activity precedes any increase in the rate of thymidine incorporation by several hours. This suggests that while increased thymidine kinase activity may be required for increased rates of thymidine incorporation, other steps are probably regulated as well.

The effects of antiestrogens may show considerable drug, tissue, and species specificity. For example, nafoxidine mimics early estrogen effects in immature rat uterus (21). Obviously, it would be of particular interest to know whether antiestrogens are ever stimulatory of human breast cancer. The results shown in figure 2 suggest that at 10^{-7} M, tamoxifen exerts no stimulatory effect at any time studied. The inhibitory effects of two antiestrogens, CI-628 and tamoxifen, are shown as a function of dose in figure 3. Both inhibit nucleoside incorporation equivalently. Inhibition is first seen at a concentration of about 10^{-8} M and is maximal at 10^{-6} M. No concentration tested is stimulatory. We have previously shown (14) that tamoxifen does not inhibit several human breast cancer cell lines in tissue cultures which lack estrogen receptor (13). Thus, three lines of evidence suggest that tamoxifen inhibits cells specifically by interfering with an estrogen-stimulated pathway. First, antiestrogen effects are prevented by simultaneous incubation with 17β-estradiol even when the latter is present in far lower concentrations (fig 1 and tables 1–4). Second, antiestrogen inhibition, once established, can be reversed by subsequent addition of estrogen (fig 2). Third, antiestrogen effects are only seen in estrogen receptor-containing cell lines such as MCF-7 (12,23) and ZR-75-1 (14).

One puzzling feature of antiestrogenic effects on the MCF-7 cell line is that incubation with antiestrogen is more inhibitory than simple estrogen deprivation. In order to understand this process more fully, we studied antiestrogen interaction with estrogen receptor. In figure 4 we compare the ability of increasing concentrations of unlabeled estradiol

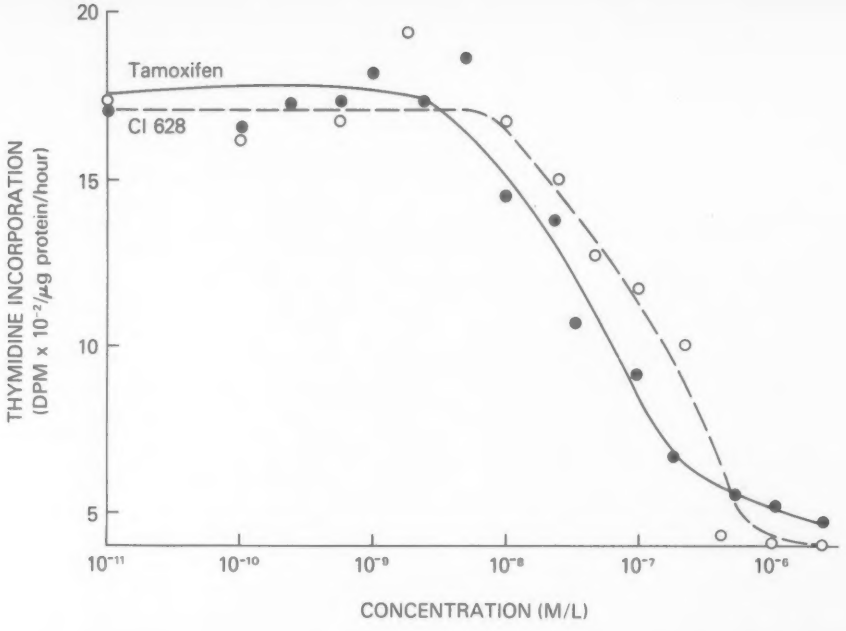

FIGURE 3.—Effects of CI-628 and tamoxifen on thymidine incorporation in MCF-7 human breast cancer. Results are means of triplicate determinations; SDs are generally < 10%.

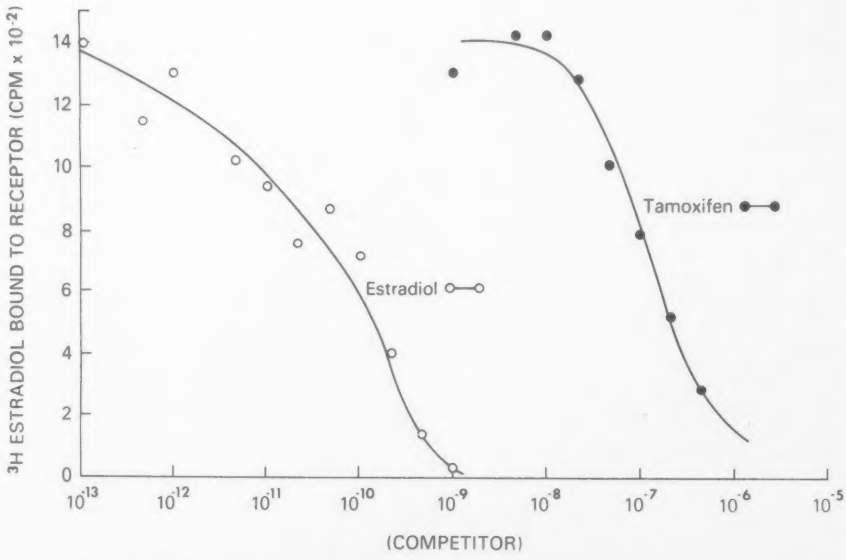

FIGURE 4.—Competition of either unlabeled 17β-estradiol or tamoxifen with ^{3}H-17β-estradiol (10^{-9} M). Techniques employed are described in the Methods section.

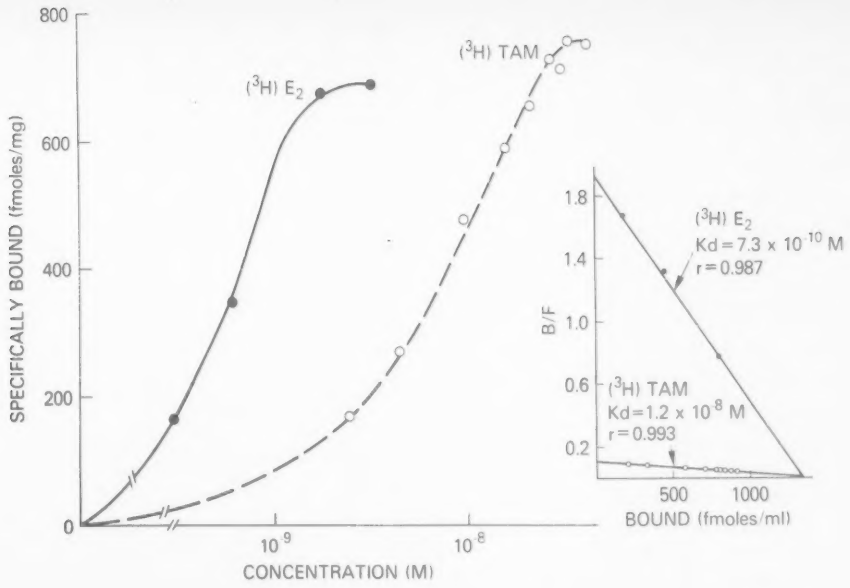

FIGURE 5.—Binding of either ^{3}H-17β-estradiol or ^{3}H-tamoxifen to rat uterine cytoplasmic extracts. E_2 = 17β-estradiol; TAM = tamoxifen. Binding data shown are replotted in the inset using the Scatchard technique as described in the Methods section.

or tamoxifen to compete with ^{3}H-estradiol for binding sites. As shown, both compete to the same extent. The tamoxifen competition curve however, is > 100–500-fold displaced to the right of the estradiol competition curve. This suggests that tamoxifen may have a far lower affinity for estrogen receptor than estradiol. It is of interest that the difference in the apparent affinities of 17β-estradiol and tamoxifen for estrogen receptor is similar to the concentration range across which estradiol can reverse antiestrogen effect. Furthermore, comparison of figures 3 and 4 reveals that there is good agreement between concentrations of antiestrogen which compete with ^{3}H-estradiol for receptor sites and concentrations which inhibit the cells.

Clearly, one could observe apparent competition by the antiestrogen if it were simply inactivating receptor or removing estrogen from nonspecific sites. This latter possibility is quite unlikely since the concentration of ^{3}H-17β-estradiol employed (10^{-9} M) is such that almost all binding is to competable sites. Therefore, such competition studies are useful but not as informative as direct binding studies. Although we have obtained a small amount of ^{3}H-tamoxifen (specific activity, 19.5 curies/mM), by the time of this presentation satisfactory binding studies had been carried out on rat uterine cytosol but not on human breast cancer cell lines. Results are shown in figure 5. ^{3}H-estradiol and ^{3}H-tamoxifen each bind to an equivalent number of binding sites at receptor-saturating concentrations of steroid. This is indicated by the common X intercepts of the Scatchard plots shown in the inset. The straight line obtained for both compounds suggests that each compound is binding to a single class of receptor sites of uniform affinity. While ^{3}H-17β-estradiol binds to estrogen receptor with about a 20-fold higher affinity than ^{3}H-tamoxifen (K_d = 1.2 × 10^{-8} M vs 7.3 × 10^{-10} M), nonetheless, these differences are smaller than those suggested by the competition studies shown in figure 4. It should be recalled that these direct binding studies are not done on the human receptor and perhaps results in that system will be different. The results in figure 1 which show that 100–1000-fold less estradiol can block antiestrogen effect suggests that the results shown in figure 4 may be more nearly correct. However, tamoxifen has a very low solubility in aqueous solutions and tends to stick to glass surfaces, and it is possible that the results shown in figures 1 and 4 reflect an artifactually lower tamoxifen concentration than anticipated.

If tamoxifen acts by competing with estrogen for receptor sites, it is difficult to understand how tamoxifen alone can inhibit cells to a greater extent than cells grown in control, hormone-free medium.

We have already reviewed the data establishing the specificity of the tamoxifen effect (prevention or reversal by estradiol, and absolute requirement for estrogen receptor). Furthermore, we have shown that all antiestrogens we have studied, tamoxifen, nafoxidine, CI-628, and clomiphene citrate, similarly inhibit cells below control levels. We believe that antiestrogens do not act by simply competing with estrogen for receptor sites. Studies by others (10,11,21) have clearly demonstrated that antiestrogen associated with receptor can translocate to nuclear sites. Nuclear binding of the antiestrogen complexes has been shown to be quite different from estrogen-receptor complexes by criteria of nuclear retention time and salt extractability. We hypothesize that in our human breast cancer cell lines in culture, important mRNA transcripts coding for growth regulatory proteins are synthesized at a rate sufficient to support slow cell growth in defined hormone-free media. When estradiol is added, estrogen-receptor complexes translocate to specific nuclear sites and increase synthesis of these key mRNA molecules. An increase in initiation sites by purified steroid-receptor complexes has been shown for the chick oviduct system (24). The qualitative alterations in mRNA production result in increased cell proliferation. In contrast, we suggest that antiestrogen-receptor complexes can inhibit synthesis of these regulatory mRNA molecules below control levels and perhaps to a level incompatible with cell viability. It should be borne in mind that cells must undergo extensive adaptions to in vitro tissue culture conditions and that this process is undoubtedly strongly selective. Furthermore, our observations are made on malignant cells, not normal tissues, such as oviduct or uterus. Nonmalignant tissues in vivo may not show further inhibition by antiestrogen as compared to hormone deprivation because of their nonproliferative state. In normal tissues there may be critical differences between cells in hormone-primed tissues. Thus, following castration in many adult female species, one observes rapid regression of estrogen-dependent tissue such as the uterus. This is largely accomplished by cell death and loss of tissue mass. Many cells in the uterus are, therefore, absolutely estrogen dependent and die without estrogens. Once total regression has occurred, the tissue may be restimulated by exogenous estrogen administration. Thus, there is a second component of estrogen-dependent tissue for which estrogens may be considered to be permissive. Obviously these cells are able to maintain themselves without steroid but are capable of estrogen response. Effects of antiestrogens may depend in part on the degree to which steroid hormones in a given tissue are either permissive for growth or absolute requirements for survival. If, indeed, there are certain estrogen-regulated RNA or protein products which control cell proliferation, their elucidation may add much to our ability to control the rate of growth of hormone-responsive tumors.

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Phase II Study of Tamoxifen: Report of 74 Patients With Stage IV Breast Cancer[1]

Harvey J. Lerner,[2,*] Pierre R. Band,[3,4] Lucien Israel,[5] and Benjamin S. Leung[6,7]

SUMMARY

Tamoxifen (NSC-180973), a synthetic antiestrogen, was studied for efficacy and toxicity in patients with metastatic breast adenocarcinoma. Two dose levels were used, 10 mg bid and 15 mg/m² bid, in separate groups. In the 10-mg bid dosage group, 30 of the 31 patients were considered evaluable for efficacy. Five complete and 11 partial responses were recorded, for an overall response rate of 53%. In the 15-mg/m² bid dosage group, 44 of the 45 patients were considered evaluable for efficacy. Three complete and 16 partial responses were recorded, for an overall response rate of 43%. All 76 patients were evaluated for toxicity. Side effects were generally mild, consisting mostly of hot flushes, transient leukopenia, transient thrombocytopenia, nausea, and fluid retention. A high degree of correlation between response and positive estrogen-receptor assay suggests the value of the test as a means to select patients for tamoxifen treatment. The conclusion from this study is that tamoxifen used as a single agent is an effective drug with minimal toxicity for treatment of metastatic breast adenocarcinoma.

[Cancer Treat Rep 60:1431–1435, 1976]

Tamoxifen is a synthetic triphenylethylene derivative with potent antiestrogen activity in several mammalian species (1). It inhibits the binding of estradiol to uterine receptors from various experimental animals (2) and exerts an inhibitory effect on the growth of the DMBA-induced rat mammary tumor (3). In man, tamoxifen inhibits the binding of estradiol to receptors from normal endometrium (4) and adenocarcinoma of the breast and uterus (5,6). It has been shown to possess therapeutic activity against metastatic breast cancer (7–9).

This report presents the results of a joint phase II clinical study of tamoxifen in 76 patients with advanced breast cancer conducted in three major institutions.

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[2]Pennsylvania Hospital, Philadelphia.

[3]University of Alberta Medical School, Edmonton, Canada.

[4]*Present address:* Institut du Cancer de Montreal, Seitre hospitaler Notre-Dame, Canada.

[5]C. H. U. Lariboisiere, Paris, France.

[6]University of Oregon Medical School, Portland.

**Reprint requests to:* Harvey J. Lerner, MD, Department of Surgery, Pennsylvania Hospital, Philadelphia, Pa 19107.

MATERIALS AND METHODS

All patients reported in this series were women with evaluable disease. Except for nine patients, all had undergone prior mastectomy and approximately half had at least one course of non-hormonal chemotherapy.

The criteria for patient inclusion were: histologically proven breast adenocarcinoma with metastases; a minimum expected survival time of 4 months; documented disease progression, particularly following discontinuation of prior additive hormonal therapy; and normal liver and renal function tests. However, patients with abnormal liver function tests due to liver metastases were included.

All patients were stratified according to dominant metastatic site (visceral, osseous, or soft tissue) and menopausal status at the time of entry. All but three patients were postmenopausal.

Responses were classified as either complete or partial. Criteria for a complete response were the complete disappearance of all measurable lesions and recalcification of osteolytic lesions with appearance of no new lesions. Criteria for partial response were a decrease of at least 50% in the sum of the products of the two largest perpendicular diameters

of all measurable lesions with at least disease stabilization in all other evaluable sites and partial recalcification of osteolytic lesions or stabilization of osteoblastic lesions, if present.

Baseline studies included tumor measurements, complete blood counts including differential white blood cell and platelet counts, liver and renal function tests, serum calcium, chest roentgenogram, and skeletal survey. In the case of a negative skeletal survey, a bone scan was performed. Tumor measurements and blood counts were repeated every 2 weeks, serum calcium and liver and renal function tests every month, and skeletal survey or bone scan every 2 months.

Two dose levels of tamoxifen, which were not randomly allocated, were studied. Thirty-one patients were treated with a dose of 10 mg bid and 45 patients were treated with a dose of 15 mg/m² bid. The latter dose is about 2½ times the lower dose since 10 mg is approximately equal to 6 mg/m².

Treatment was designed to be given for 16 weeks (twice the average clinical doubling time of breast carcinoma) or until there were signs of disease progression or treatment-limiting toxicity. If no change had occurred, or if there was regression at the end of the 16-week period, treatment was continued until disease progression or relapse. Patients were considered evaluable for efficacy if they completed at least 4 weeks of therapy. All patients treated were considered evaluable for toxicity regardless of the time they received tamoxifen.

Prior to therapy with low doses of tamoxifen,

TABLE 1.—Tamoxifen (10 mg bid): patient characteristics and therapeutic results

Patient—							Response		
No.	Age	Yrs post-menopausal	Disease-free period (yrs)	Disease sites*	Prior therapy†	Treatment duration‡ (days)	Type§	Sites*	Duration of CR + PR‡ (days)
Soft tissue dominant									
1	29	Premenopausal	1–5	SK	S	362	CR	SK	286
2	60	> 5	> 5	ST, ND	S-H-C	390	CR	ST, ND	338
3	60	> 5	< 1	ST	S	106	NC	—	—
4	61	> 5	< 1	SK	S-C	10	PROG	—	—
5	75	> 5	> 5	ND	S-C	574+	CR	ND	554+
6	76	> 5	< 1	ND	S	283	CR	ND	175
7	79	> 5	< 1	SK, ST	None	522+	PR	SK, ST	470+
8	80	> 5	1–5	SK, ST	S-C	428+	CR	SK, ST	366+
9	84	> 5	< 1	ST, ND	None	134	PR	ST, ND	42
Osseous dominant									
10	46	Premenopausal	> 5	ND, BN	S	120+	NC	—	—
11	50	1–5	1–5	BN	S-RT	123	NC	—	—
12	60	> 5	> 5	BN	S-RT	159	PROG	—	—
13	65	> 5	1–5	BN	S	397	PR	BN	367
14	66	> 5	< 1	ST, BN	S-RT	23	PROG	—	—
15	78	> 5	< 1	ST, BN	S-RT	547+	PR	ST, BN	509+
Visceral dominant									
16	47	1–5	1–5	SK, PL	S-RT-H-C	107	PROG	—	—
17	53	1–5	< 1	ST, LG	S-C	636	NC	—	—
18	54	1–5	1–5	BN, LG	H-S-RT-C	92	NC	—	—
19	58	> 5	> 5	ST, LG	H-S-RT-C	557+	PR	ST, LG	384
20	60	> 5	1–5	ND	S-RT-C	15	PROG	—	—
21	60	> 5	< 1	SK, BN, LG	S-C-RT	64	PROG	—	—
22	59	> 5	< 1	(BN),¶ LG	S-C	132	PR	LG	51
23	60	> 5	< 1	ST, (BN),¶ LV, LG	RT	286+	PR	ST, LV, LG	239+
24	62	> 5	< 1	LG	S-C	114	PROG	—	—
25	61	> 5	1–5	LG	S-C	554	PR	LG	277
26	61	> 5	< 1	SK, PL	S-H-C	205	PR	SK	66
27	61	> 5	> 5	ST, BN, LG	S	404+	PR	ST, LG	343+
28	63	> 5	< 1	ND, LV	S-C	252	PROG	—	—
29	65	> 5	1–5	LG	S-C	253	PR	LG	161
30	82	> 5	< 1	BN, PL	S-RT	304	NC	—	—

*AS = ascites; BN = bone; LG = lung; LV = liver; ND = lymph nodes; PL = pleura; PT = peritoneum; SK = skin; ST = soft tissue.
†S = surgery; RT = radiation therapy; H = hormonal therapy; C = chemotherapy.
‡Plus sign indicates that the patient is still on study.
§CR = complete response; PR = partial response; NC = no change; PROG = progression.
¶Nonevaluable due to concomitant X-ray therapy.

tumor samples were taken from 17 patients in the operating room, immediately placed in liquid nitrogen, and transferred to the University of Oregon Health Science Center laboratory for estrogen-receptor (ER) measurements using the dextran-coated charcoal assay method previously described (10). In addition, 5 mM of dithiothreitol was added to the buffer system to better preserve ER. Using this method, tumors with estrogen-binding sites > 1 fmol/mg of cytoplasmic protein are considered to be ER positive (10).

RESULTS

Tamoxifen at 10 mg bid

Of the 31 patients receiving this dose, 30 were evaluable for efficacy. One patient was considered ineligible because of nonevaluable disease (pleural effusion only). Three patients died during treatment due to unrelated progressive disease. One patient died of a cardiovascular accident while in complete remission after 390 days of therapy, one patient died of a myocardial infarction while in partial remission after 205 days of therapy, and one patient died of a pulmonary embolism after 10 days of therapy. Patient characteristics and observed toxicity are described in tables 1 and 2 respectively.

Five complete and 11 partial responses were recorded, for an overall response rate of 53%. The median duration of response was 312+ days. Response of soft tissue lesions occurred in 12 patients. Recalcification of osteolytic lesions occurred in two patients. Regression of pulmonary metastases occurred in six patients and a liver response was recorded in one patient. The median age of the responders was 63 years (range, 29–84) compared with 60 years (range, 46–82) for nonresponders. Side effects were generally tolerated and did not require drug discontinuation. Leukopenia or thrombocytopenia, observed in ten patients, spontaneously reverted to normal despite continuous drug administration without dosage reduction. Hot flushes, the most common toxic effect, tended to decrease with time. One patient required dosage reduction for severe bone pain. No further problem occurred when the dosage was returned to the original level.

TABLE 2.—Toxic effects from tamoxifen

Side effects	No. of cases	
	10 mg bid	15 mg/m² bid
None	12	25
Hot flushes	15	8
Leukopenia	6 (nadir, 2800 cells/mm³)	5 (nadir, 3000 cells/mm³)
Thrombocytopenia	4 (nadir, 80,000 cells/mm³)	3 (nadir, 70,000 cells/mm³)
Edema	—	3
Nausea	1	3
Vaginal discharge	2	2
Vaginal bleeding	1	—
Alopecia	—	1
Other	—	1 (nausea, vomiting, headache, vertigo, epigastric pain)

Tumors from 17 patients were assayed for ER before the start of tamoxifen therapy. Thirteen tumors were ER positive (> 1 fmol/mg protein) and four were ER negative (< 1 fmol/mg). Of the patients with ER-positive tumors, nine responded to tamoxifen therapy, two had disease progression, and two remained stable. Of the patients with ER-negative tumors, three had disease progression and one remained stable (table 3).

TABLE 3.—Tamoxifen (10 mg bid): correlation of response with ER assay

Responders and stabilized patients				Nonresponders		
Patient No.	ER (fmol/mg protein)	Response*	Site of response	Patient No.	ER (fmol/mg protein)	Response*
18	0.0	NC	Lung	28	0.2	PROG
6	3.9	CR	Soft tissue	21	0.5	PROG
3	7.6	NC	Soft tissue	16	0.86	PROG
1	9.5	CR	Soft tissue	14	1.60	PROG
10	10.8	NC	Soft tissue	4	54.0	PROG
23	13.6	PR	Lung			
9	17.5	PR	Soft tissue			
2	19.6	CR	Soft tissue			
5	22.8	CR	Soft tissue			
22	25.5	PR	Lung			
8	35.6	CR	Soft tissue			
27	36.0	PR	Soft tissue			

*CR = complete response; PR = partial response; NC = no change; PROG = progression.

TABLE 4.—Tamoxifen (15 mg/m² bid): patient characteristics and therapeutic results

Patient—							Response		
No.	Age	Yrs post-menopausal	Disease-free period (yrs)	Disease sites*	Prior therapy†	Treatment duration‡ (days)	Type§	Sites*	Duration of CR + PR‡ (days)
Soft tissue dominant									
1	45	Premenopausal	< 1	SK, ST, ND	RT	400+	PR	SK, ST, ND	381+
2	52	< 1	< 1	ND	S	56	NC	—	—
3	36	> 5	1–5	SK, ND	S-RT-H	36	PROG	—	—
4	55	> 5	1–5	SK	S-RT-H-C	271+	CR	SK	226+
5	56	> 5	< 1	ST, ND	RT-H	612+	PR	ST, ND	584+
6	55	> 5	1–5	SK, ND	S-RT	117	PR	SK, ND	89
7	59	> 5	1–5	SK	S-RT	323+	PR	SK	148+
8	61	> 5	1–5	SK, ND	S-RT-C	168	NC	—	—
9	61	> 5	> 5	SK	S-H	161	PR	SK	134
10	68	> 5	1–5	ST	S	78	PROG	—	—
11	73	> 5	1–5	ST	RT-C	234+	PR	ST	190+
Osseous dominant									
12	51	< 1	< 1	SK, ND, BN	S	50	PROG	—	—
13	32	1–5	< 1	BN	S-RT-C	128	PR	BN	86
14	44	1–5	1–5	BN	S-RT-H	76	PR	BN	43
15	46	1–5	1–5	BN	S-H-C	77	NC	—	—
16	56	1–5	1–5	BN	S-RT-H	494+	PR	BN	364+
17	59	1–5	1–5	BN	S-RT-C	97	PROG	—	—
18	47	> 5	< 1	SK, ND, BN	S-RT-H-C	300+	NC	SK, ND	—
19	56	> 5	< 1	ND, BN	S	17	PROG	—	—
20	58	> 5	1–5	BN	S-H	170	PR	BN	98
21	60	> 5	< 1	SK, ND, BN	RT-H-C	63	PROG	—	—
22	63	> 5	1–5	BN	S-RT-C-H	424+	PR	BN	326+
23	66	> 5	1–5	BN	S-RT	50	PROG	—	—
Visceral dominant									
24	56	< 1	< 1	ST, BN, LG	S-RT-H	452+	PR	ST, BN, LG	323+
25	40	1–5	1–5	BN, LG	S-RT-C-I	257	PR	BN, LG	229
26	41	1–5	1–5	BN, LG	S-RT-H-C	218	PROG	—	—
27	43	1–5	1–5	SK, PL	S-RT-H-C	57	PROG	—	—
28	44	1–5	1–5	ST, LG, BN	H	84	PROG	ST	—
29	48	1–5	1–5	SK, PL	S-RT-C	542+	CR	SK, PL	500+
30	56	1–5	1–5	LG	S-RT-C	50	PROG	—	—
31	48	> 5	1–5	BN, PL	S-RT-H-C	406+	PR	BN	330+
32	54	> 5	> 5	ST, BN, AS	S-RT-C	36	PROG	—	—
33	52	> 5	> 5	LG	S-H-RT	66	NC	—	—
34	55	> 5	1–5	BN, PL	RT-H-C	29	PROG	—	—
35	58	> 5	1–5	LG	S-RT	111	NC	—	—
36	59	> 5	1–5	BN, LG	S-RT-C	43	PROG	—	—
37	61	> 5	1–5	SK, LG, LV, PL, PT	S-RT-H-C	113	NC	—	—
38	63	> 5	1–5	ND, BN, LG, LV, PL	S-RT-H-C	95	PROG	—	—
39	65	> 5	1–5	SK, PL	S-RT-C	282	CR	SK, PL	225
40	68	> 5	> 5	ND, PT	S-H-C-I	34	PROG	—	—
41	70	> 5	1–5	ND, LG, LV	S-RT	67	NC	—	—
42	70	> 5	1–5	BN, LG	S-C	418+	PR	BN, LG	311+
43	76	> 5	1–5	LG	S-RT	169	PROG	—	—
44	79	> 5	> 5	BN, LG	S	325+	PR	BN, LG	220+

*AS = ascites; BN = bone; LG = lung; LV = liver; ND = lymph nodes; PL = pleura; PT = peritoneum; SK = skin; ST = soft tissue.
†S = surgery; RT = radiation therapy; H = hormonal therapy; C = chemotherapy.
‡Plus sign indicates that the patient is still on study.
§CR = complete response; PR = partial response; NC = no change; PROG = progression.

TABLE 5.—Response in relation to dominant site of disease

Dominant site	Responders (CR + PR)/No. of patients		
	10 mg bid	15 mg/m² bid	Both regimens
Soft tissue	7/9 (78%)	7/11 (64%)	14/20 (70%)
Bone	2/6 (33%)	5/12 (42%)	7/18 (38%)
Visceral	7/15 (47%)	7/21 (33%)	14/36 (39%)
Total	16/30 (53%)	19/44 (43%)	35/74 (47%)

Tamoxifen at 15 mg/m² bid

Of the 45 patients receiving this dose, 44 were evaluable for efficacy. One patient was considered ineligible because of two primary sites (ovary and breast). All 45 patients were evaluated for toxicity.

Observed toxicity and patient characteristics are described in tables 2 and 4 respectively. Three complete and 16 partial responses were observed, for an overall response rate of 43%. The median duration of response was 227+ days. Response of soft tissue lesions occurred in 12 patients. Recalcification of osteolytic lesions occurred in ten patients and regression of pulmonary metastases occurred in three. Also, pleural effusions in two patients were resolved. The median age of the responders was 56 years (range, 32–79) compared with 58 years (range, 36–76) for the nonresponders.

Side effects were generally mild, consisting mostly of hot flushes, transient leukopenia, transient thrombocytopenia, nausea, and fluid retention. In one patient, however, nausea, vomiting, headaches, epigastric pain, and vertigo developed. These symptoms disappeared when tamoxifen was withdrawn but recurred following reinstitution. Treatment was therefore discontinued for this patient. One patient required dosage reduction for severe bone pain. No further problem occurred, however, when the dosage was returned to the original level.

DISCUSSION

These data indicate that tamoxifen is an effective drug in the treatment of advanced breast cancer, confirming results obtained by other investigators (7–9).

Response rates were similar in both treatment regimens in relation to dominant site (table 5) and did not differ across postmenopausal categories (tables 1 and 4).

Side effects occurred in approximately 50% of the patients, were generally well tolerated, and did not require drug discontinuation except in one case. In particular, leukopenia or thrombocytopenia, observed in 14 patients, spontaneously reverted to normal despite continuous drug administration without dosage reduction. Hot flushes, the most common toxic effect, tended to decrease with time. With one exception, fluid retention was mild and did not require the administration of diuretics. Two patients (one at each dose level) required dosage reduction for severe bone pain. In both cases, the pain subsided at the reduced dosage. No further problems occurred when the dosage was escalated to the original dose level.

The high degree of correlation observed between response rate and positive ER assay suggests the value of this test as a means to select patients for tamoxifen treatment.

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Therapeutic Use of Tamoxifen in Advanced Breast Cancer: Correlation With Biochemical Parameters

Lee Roy Morgan, Jr,[1,*] Philip S. Schein,[2] Paul V. Woolley,[2] Daniel Hoth,[2] John Macdonald,[2] Marc Lippman,[3] Lynn E. Posey,[1] and Robert W. Beazley[1,4]

SUMMARY

Tamoxifen (NSC-180973; ICI-46474) can provide palliation to patients with advanced breast cancer whose tumors contain estrogen-binding proteins (EBP). The drug is most effective in patients with bone metastasis and minimal prior therapy. In the present study, 72 patients with advanced breast cancer were evaluated for their response to oral tamoxifen therapy administered at a dose of 20 mg twice daily. Twenty-eight of 72 patients (38%) demonstrated objective responses to tamoxifen therapy. For patients with a positive EBP and no prior chemotherapy, eight of 11 (74%) responded. No patient possessing a tumor negative for EBP achieved a remission. Patients with tumors that possessed normal arylsulfatase B and glucose-6-phosphate dehydrogenase enzyme activities responded most favorably to tamoxifen therapy. These results demonstrate that tamoxifen is effective in the treatment of patients with advanced breast cancer, and EBP and specific enzymes might be useful in selecting the patients for hormone manipulation.

[Cancer Treat Rep 60:1437–1443, 1976]

Tamoxifen, a triphenylethylene derivative, the *trans* isomer of 1-(*p*-*β*-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene citrate, is an antiestrogen that is currently undergoing clinical studies in this country in advanced breast cancer (1,2). Tamoxifen has been demonstrated to cause increased uterine weight and incomplete vaginal cornification in oophorectomized rats indicating a degree of estrogenic activity (3). However, in some species in the presence of estrogens it acts as an antiestrogen, without demonstrable estrogen activity (3). Tamoxifen has been found to inhibit the growth of DMBA-induced rat mammary tumors. It inhibits tumor binding of estradiol in vivo and reduces estrogen-stimulated blood prolactin levels. At the subcellular level tamoxifen inhibits the binding of estradiol to the 8S estrogen-binding protein (EBP) from both DMBA-induced rat mammary carcinomas and human breast carcinomas (4). In clinical studies in Europe and Great Britain, tamoxifen has been reported to produce objective clinical remissions in 22%–36% of patients with advanced breast cancer (5–7).

The present study was designed to investigate the effect of tamoxifen as an antitumor agent in advanced breast cancer. This report will describe the response of patients with metastatic breast cancer to tamoxifen and attempt to correlate the clinical responses to tamoxifen with tumor tissue content of cytoplasmic EBPs and other biochemical parameters.

PATIENTS AND METHODS

Patients

Women with advanced carcinoma of the breast were selected to receive tamoxifen. The majority of the patients possessed multiple lesions. When possi-

[1]Departments of Pharmacology, Medicine, Pathology, and Surgery, Louisiana State University Medical Center, New Orleans.
[2]Division of Medical Oncology, Vincent T. Lombardi Cancer Research Center, Georgetown University School of Medicine, Washington, DC.
[3]Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[4]The invaluable technical assistance of Laura Gillen, Donna Blistine, Lynne Harmon, and Andrea Hagardorn is gratefully acknowledged.
*_Reprint requests to:_ Lee Roy Morgan, Jr, MD, PhD, Departments of Pharmacology and Medicine, Louisiana State University Medical Center, 1542 Tulane Ave, New Orleans, La 70112.

ble, tumor tissue from either cutaneous or other metastatic sites was selected for biopsy and the remaining skin nodules or metastases or both were evaluated for response to drug therapy. Ages ranged from 27 to 82 years and one third of the patients were black. All patients were evaluated for their menopausal status: seven patients were premenopausal, 63 patients were postmenopausal, and two patients had recent endocrine ablation (< 6 months). Prior hormone therapy, radiotherapy, and chemotherapy were well documented in all patients. Patients were not admitted to the trial unless 4 weeks had elapsed since prior treatment and objective progression was documented to assure that no response to tamoxifen would have been the result of coincidental therapy, particularly radiation. All patients had histologically proven infiltrating ductal carcinoma of the breast as their primary lesion.

Treatment and Biopsy

Ten-milligram tablets of tamoxifen were given orally for a minimum of 4 weeks at a dose of 20 mg twice daily, irrespective of weight or surface area. Treatment was continued indefinitely if an objective remission was demonstrated. The patients were seen weekly for the first month of therapy and, thereafter, they were followed at monthly intervals. When possible, prior to initiating drug therapy, a biopsy specimen at least 1 cm in diameter was taken for determination of cytoplasmic EBPs, as well as for specific enzyme activities. When possible, a second biopsy for EBP, enzyme, and DNA content was obtained at least 8 weeks after therapy when objective progression or regression of the tumor was observed. Care was taken to obtain the initial biopsy of tissue outside any irradiated fields. At the beginning of the trial all patients had routine hematologic and chemical investigations in addition to roentgenographic evaluation. All patients had normal hematologic and renal function tests.

EBP, Enzyme, and DNA Assays

The method of handling specimens after biopsy, preparation of cytosol, and assay for EBP by the dextran-coated charcoal and the sucrose gradient techniques were previously reported by Leung et al (8), Wittliff et al (9), Morgan et al (10), and Lippman et al (11). Specific estrogen-binding capacity was specified as femtomole (fmol) of ^{3}H-17β-estradiol bound per mg of cytoplasmic protein (9). In this study tumors were classified as positive for EBP if the cytoplasmic estrogen-binding capacity was > 10 fmols/mg of protein. Tissue arylsulfatase B (EC 3.1.6.1) and glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49) activities and DNA values were determined according to previously described procedures (12,13). Enzyme activity for arylsulfatase B is expressed in micromoles of 4-nitrocatechol (NC) liberated per microgram of DNA [(μmols of NC/μg DNA)10^4]. Activity for G-6-PD is described as international units per microgram of DNA [(IU/μg DNA)10^4].

Criteria for Response

Complete response was defined as disappearance of all objective evidence of disease or recalcification of osteolytic lesions in measured and unmeasured sites for 12 weeks. A partial response was a decrease by > 50% in the product of the two largest perpendicular diameters of measurable lesions and/or partial recalcification of osteolytic lesions (regression or no change in osteoblastic lesions). No change indicated regression of < 50% in measured areas which lasted for at least 3 months. Progression was an increase of > 20% of measured lesions and/or the occurrence of new lesions and/or progression of osteolytic lesions.

RESULTS

Seventy-two patients were treated with tamoxifen for a period of at least 4 weeks. The overall response rate was 38% (28 of 72 patients). As indicated in table 1, most of the responders were in the postmenopausal group. One patient who had responded to an oophorectomy for a 3-month period responded to tamoxifen with a > 50% reduction in skin nodules.

Table 2 is an evaluation of the age of our patients with a more definitive description of their menopausal status and response to therapy. The patients who responded to the greatest extent were in the > 70-year age group and those with the greatest number of years after the menopause.

Durations of response are listed in table 3. The present study has been in progress for only 14 months. However, one of the patients in our study has been receiving therapy for 18 months after being referred to one of us (Dr. Schein). At present the median duration of response is 9½ months for the patients in the study. Seven patients who have been receiving therapy for at least 3 months remain in remission. Four of our complete responders have been in a continuous remission for ≥ 14 months.

Table 4 analyzes response rate in relation to specific organ sites of evaluable disease. In our study, the predominant lesion was bony metastasis; 21 of 28 responders had bony involvement which responded to therapy as demonstrated by recalcification. Cutaneous lesions were the next most fre-

TABLE 1.—Responders to tamoxifen according to menstrual status

Status	No. of patients	No. of responders*			
		CR	PR	NC	NR
Premenopausal	7	1			6
Postmenopausal†					
0–5 yrs	18	2	4	1	11
> 5 yrs	45	4	12	3	26
Postablation (< 6 mos)	2	—	1	—	1
	72	7	17	4	44
		28 (38%)			
		24 (33%)			

*See text for definitions of response.
†Postmenopausal: ≥ 55 yrs old and/or < 6 mos postoophorectomy.

TABLE 2.—Age, yrs menopausal, and response

All patients			Postmenopausal patients		
Age (yrs)	No. of patients	No. of responders	Yrs postmenopausal	No. of patients	No. of responders
< 40	6	2	< 5*	20	8
40–50	16	7	5–10	28	6
51–60	27	7	11–15	17	7
61–70	17	8	> 15	6	5
> 70	6	4			

*Postablation included.

TABLE 3.—Duration of response to tamoxifen

Duration of response* (mos)	No. of patients
< 3	7
3–6	5
7–12	15
13–18	1

*Median duration = 7.5 mos.

TABLE 4.—Organs involved: responders vs nonresponders

	No. of patients with involvement of—			
	Bone	Viscera	Lymphatics	Cutaneous tissue
Responders (28 patients)	21	4	3	9
Nonresponders (44 patients)	26	23	16	25

quent site of involvement, with visceral and lymphatic being less prevalent. The four responders with visceral involvement all had nodular pulmonary metastasis. No responses were demonstrated in six patients with liver metastasis. A specific pattern of response was observed in patients with cutaneous lesions that were EBP positive. Initially, tumors became less pruritic and painful, followed by a mild erythema and edema, especially around the peripheries. This was sometimes followed by marked reduction of tumor size. Histologically, replacement with fibrous connective tissue with reduction of tumor cells was observed in clinically regressing or healed lesions.

The clinical toxicities observed with tamoxifen therapy are presented in table 5. They are comparable to experiences reported by British and European investigators. Four patients developed mild transient thrombocytopenia between Days 10 and 14 of therapy. In all cases, this was quickly reversed to a normal range despite continued therapy without clinical evidence of bleeding. Seven patients developed hot flashes during therapy which were controlled with nonphenothiazine tranquilizers. Six patients experienced nausea and one of the patients demonstrated significant vomiting which resolved after discontinuation of therapy. Four patients with osseous metastasis complained of a significant increase in bone pain. One patient had documented hypercalcemia which reversed after withdrawal

from therapy. One patient experienced a significant increase in bone pain which subsided with continued treatment; the patient then achieved a partial response. Three patients with prior oophorectomies with intact uterus demonstrated breakthrough bleeding after several weeks of treatment. This subsided in all patients despite continued therapy. All premenopausal women developed irregularity of their menstrual cycle, and three of the patients had cessation of menstrual flow. Four patients developed erythematous skin reactions; weight gain or high blood pressure was not observed in any patient. Cataracts did not develop in any patient nor was there deterioration of pre-existing ocular disease.

TABLE 5.—Side effects in patients receiving tamoxifen

Effect	No. of patients
Thrombocytopenia (90,000–125,000 platelets/mm³)	4
Hot flashes	7
Tumor pain	4
Vaginal bleeding	3
Menstrual irregularity	5
Gastrointestinal	6
Skin rash	4

Table 6 presents an analysis of the influence of previous therapy on response to tamoxifen. Only one of the 18 patients who had prior chemotherapy responded. Unfortunately, most of this group had extensive disease and were refractory to treatment, which could explain the poor response.

Figure 1 is a bar graph depicting the relationship of response to treatment and EBPs, arylsulfatase B activity (a hydrolytic enzyme present in carcinoma of the breast), and G-6-PD activity (an endoplasmic reticulum enzyme also present in human breast cancer tissue) (10,13). Enzyme activity is expressed per microgram of DNA, thereby normalizing the data to account for differences in cellularity between normal and abnormal tissues. The shaded values represent the normal ranges obtained from 23 normal breast tissue samples from nonlactating women.

TABLE 6.—No. of patients with prior therapy

	No. of patients receiving—			
	No prior therapy	Radiation therapy	Hormone therapy	Cytotoxic chemotherapy
Responders (28 patients)	3	20	9	1
Nonresponders (44 patients)	6	29	19	17

The first column in each pair of bar graphs represents values from all tissue specimens assayed. There was a 44% correlation between patients with a positive EBP assay and response to therapy. Fifteen of the 31 original patients who had no prior chemotherapy were evaluated as a group and 73% achieved a remission. In neither group did EBP-negative patients respond to tamoxifen. When the arylsulfatase B and G-6-PD activities from the same tissue were evaluated, a similar correlation between response to therapy and the number of patients with normal arylsulfatase B/DNA and G-6-PD/DNA ratios was observed. This correlation improved when patients without prior chemotherapy were analyzed separately. Again, as we have seen previously, patients with normal arylsulfatase B/DNA ratios in the tumor tissue responded better to hormonal therapy (10).

In figure 2, the relationship of response to positive EBP and enzyme activities is analyzed according to menopausal status. The one patient in the postablation period (< 6 months) is not included. Although the number of premenopausal cases are few, the percentage correlations are about the same for EBP values and arylsulfatase B activities for the two clinical groups. There was a 40% and a 63% correlation between response and the presence of a normal G-6-PD/DNA ratio in the premenopausal and postmenopausal patients respectively.

DISCUSSION

According to the current concept of estrogen action in estrogen target cells, as reviewed by Jensen and DeSombre (14), growth of sensitive tumors would require a significant level of estrogen, functional estrogen-receptor sites that are able to bind to estrogens, and an operative pathway distal to initial binding. In such an instance, an antiestrogen which effectively competes with estrogen at the estrogen-receptor sites might achieve the desired therapeutic effect.

Tamoxifen is a synthetic nonsteroidal drug, the *trans* isomer of 1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene citrate. The *cis* form has been reported to possess potent estrogenic activities while tamoxifen has antiestrogen properties (1). Tamoxifen does inhibit the in vitro binding of ^{3}H-17β-estradiol to 8S breast tumor EBP (4). This is currently the mechanism of action for the use of tamoxifen in advanced human breast cancer.

Our studies with tamoxifen clearly suggest that patients with positive EBP values respond better to the drug. Postmenopausal women with low estrogen levels had the best responses to tamoxifen therapy. Treatment of actively menstruating women pro-

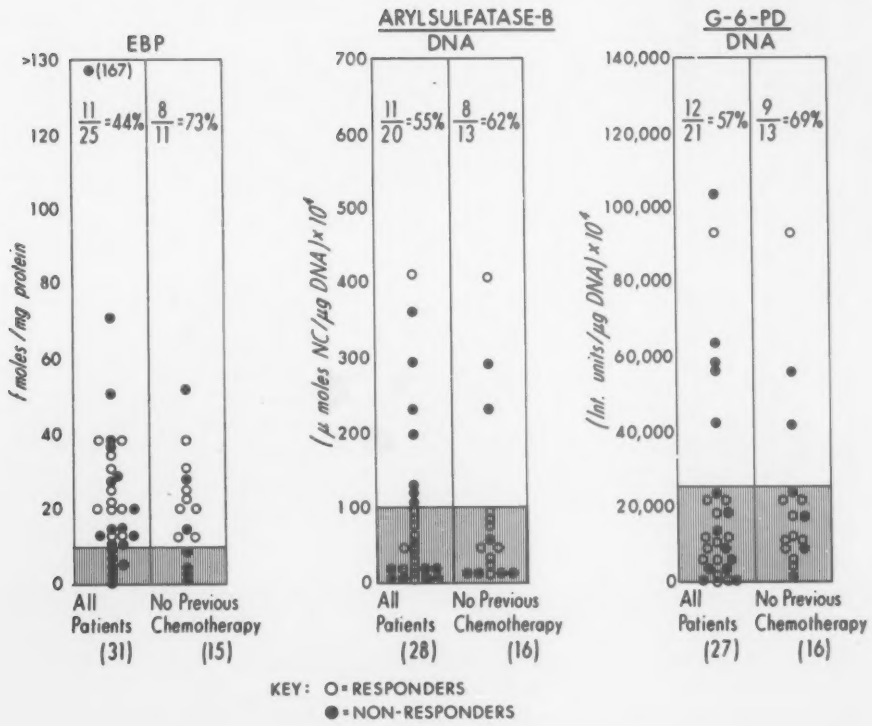

FIGURE 1.—Tissue EBP values, arylsulfatase B/DNA ratios, and G-6-PD/DNA ratios for human breast cancer compared to response. Shaded areas represent values from 23 specimens of normal breast tissue. Percentages are for patients with elevated EBP values and normal arylsulfatase B/DNA and G-6-PD/DNA ratios.

duced the most side effects with minimal response to therapy.

Tamoxifen appears to be more effective in cutaneous and bony disease than in visceral disease, especially liver metastases, which uniformly failed to respond to the drug.

The observation that patients who had had prior chemotherapy or long-standing disease with positive EBP responded less favorably to tamoxifen might be partly due to the presence of multi-clonal populations of cells in which the EBP-negative cells outnumber the positive cells in various sites. Such a diversity could explain why some patients experience remission of the disease at some sites and not in others, and why the initial regression of positive EBP tumors is usually followed by a recurrence when treated with hormone therapy alone. At the time of evaluation, the patients in this study who relapsed did so in previously uninvolved sites; the original lesions remained unchanged or in regression.

The results in the present work confirm our previous finding that patients with breast cancer who respond to hormone manipulation have a normal arylsulfatase B/DNA ratio (10). In the present study, 20 of 28 patients had normal arylsulfatase B/DNA ratios in their tumor tissue and 11 of the 20 patients with normal arylsulfatase B/DNA ratios responded to tamoxifen therapy. When this group is divided into patients with no prior therapy, eight of

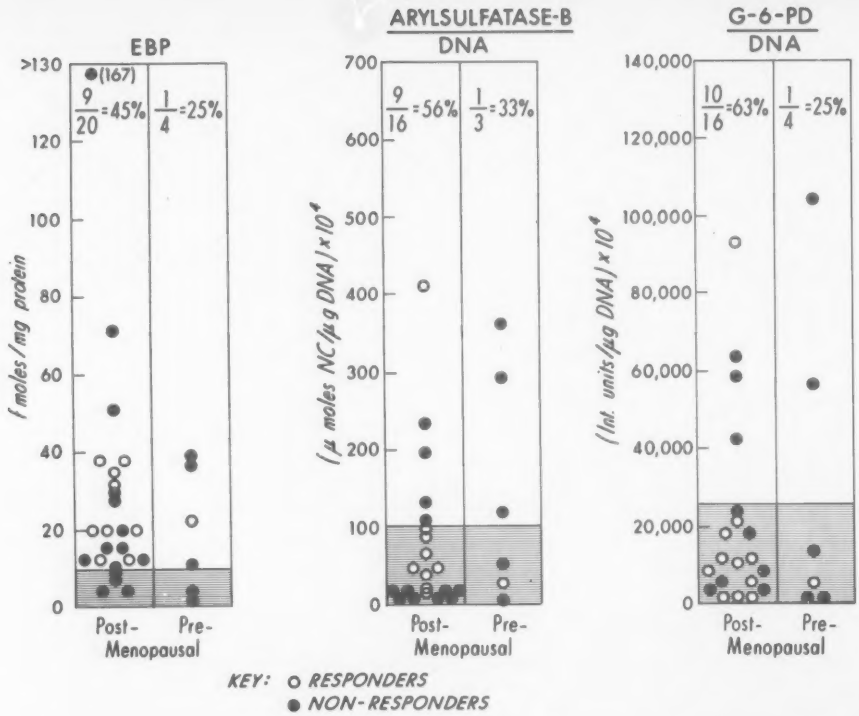

FIGURE 2.—EBP values and arylsulfatase B/DNA and G-6-PD/DNA ratios for breast cancer tissues from premenopausal and postmenopausal patients. Shaded areas represent normal values. Figures are calculated for tissues with elevated EBP values, normal arylsulfatase B/DNA ratios, and normal G-6-PD/DNA ratios as discussed in the Results.

13 patients with normal arylsulfatase B/DNA values achieved a remission. Patients who responded most favorably to therapy possessed tumor G-6-PD activities comparable to normal nonlactating breast tissue. Savlov et al (13) have also observed this and have speculated that the low G-6-PD activity in patients with positive EBPs is a reflection of the low metabolic activity of the tumor. The normal arylsulfatase B/DNA ratios in our tissue might be a similar reflection of low metabolic activity (13). Further studies are obviously needed to clarify the diagnostic and prognostic uses of these enzymes along with EBP in the evaluation of patients with early breast cancer for possible hormone palliative manipulations.

The place of antiestrogens in the treatment of carcinoma of the breast remains to be established. Tamoxifen's use, alone or with chemotherapy at the time of first recurrence after oophorectomy, is being studied by several groups. Results from these studies may provide further understanding of the antiestrogen action and a means for the rational palliation of carcinoma of the breast. It is possible that tamoxifen may obliterate the need for ablative therapy, adrenalectomy, or hypophysectomy in postmenopausal patients with positive EBPs who had a prior response to additive hormonal treatment.

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Antiestrogen-Induced Remissions in Stage IV Breast Cancer[1]

A. Manni,* J. Trujillo, J. S. Marshall, and O. H. Pearson[2,3]

SUMMARY

Tamoxifen (NSC-180973, ICI-46474), an antiestrogen, was administered to 39 women with stage IV breast cancer at a dose of 20 mg orally every 12 hours. Patients were selected as eligible for endocrine ablative treatment and with disease not so aggressive as to jeopardize further treatment in case the experimental drug failed. Objective remission was obtained in 19 patients (49%) with a mean duration of 11+ months and ten patients are still in remission. No progression was seen in seven patients (18%) lasting 13+ months with only one patient in relapse. Thirteen patients (33%) have failed. Objective remission was obtained in two premenopausal women even though menstrual cycles were not suppressed; bilateral oophorectomy in one of these patients induced a second remission after relapse from tamoxifen. Objective remissions were obtained in two women with proven complete hypophysectomy suggesting a direct action of antiestrogens at the tumor level. Positive estrogen receptors were suggestive of being a good predictor of response. Menopausal status and dominant site of metastasis did not affect the response to tamoxifen in this small series. Tamoxifen did not alter prolactin secretion, and side effects from the drug were usually mild and transient in nature. We conclude that tamoxifen is an effective antitumor agent in patients with stage IV breast cancer; further studies are necessary to determine whether it will equal the therapeutic effect of oophorectomy, adrenalectomy, and hypophysectomy.

[Cancer Treat Rep 60:1445–1450, 1976]

Potent nonsteroidal antiestrogen drugs have been developed which presumably act by competitive binding to the cytosol estrogen receptor. Clinical trials of such drugs in patients with metastatic breast cancer have been carried out to evaluate their potential usefulness as possible substitutes for endocrine ablative procedures such as ovariectomy, adrenalectomy, or hypophysectomy. Two drugs, tamoxifen and nafoxidine, have undergone clinical trials in Europe (1–4) and have been shown to induce objective tumor regression in 25%–40% of patients with advanced breast cancer. This report presents our preliminary results with the use of tamoxifen in 51 women with stage IV breast cancer.

MATERIALS AND METHODS

Thirty-nine patients with progressive metastatic breast cancer were selected for a trial of tamoxifen because they appeared to be suitable candidates for endocrine ablative treatment but their disease did not appear to be so aggressive as to potentially jeopardize a response to subsequent ablative treatment if the experimental drug failed. Patients who had progression of disease after what appeared to be an adequate trial of tamoxifen would be advised to undergo oophorectomy or hypophysectomy, whichever procedure was appropriate. Patients who obtained a remission from tamoxifen were continued on the drug until relapse occurred at which time oophorectomy or hypophysectomy was recom-

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[2]Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio.

[3]We wish to thank Lois Trench for the generous supply of tamoxifen through ICI, United States, Inc.

**Reprint requests to:* Andrea Manni, MD, University Hospitals of Cleveland, University Circle, Cleveland, Ohio 44106.

mended to determine whether further benefit would be obtained with these ablative procedures. Informed consent was obtained from all patients in this study. In all patients who had tumors which were readily accessible for biopsy, specimens were obtained for measurement of estrogen receptors, but this information was not used in selecting patients for the trial of tamoxifen. Twelve patients who had previously undergone hypophysectomy or adrenalectomy were treated with tamoxifen to determine whether further benefit might be obtained.

Tamoxifen was given at a dose of 20 mg orally every 12 hours. This dose was selected on the basis of dose-response studies by Ward (5).

Serum prolactin was measured in eight patients by homologous radioimmunoassay (6) before and at various periods after administration of tamoxifen to determine whether antiestrogen treatment influenced the secretion of prolactin.

RESULTS

Table 1 shows the results of tamoxifen therapy in the first 39 patients. Nineteen patients had objective remissions. This is defined as > 50% reduction in the size of measurable dominant lesions or recalcification of osteolytic lesions with no progression elsewhere and no new lesions appearing. The mean duration of the remissions is 11+ months with ten of the 19 patients still in remission at the time of this report. Seven patients had no progression of their disease, lasting an average of 13+ months, with only one of these patients having shown progression after a period of 9 months. Thirteen patients failed to respond to tamoxifen.

Table 2 shows the menopausal status versus response in this group of 39 patients. Ages ranged from 27 to 80 years. Two patients were premenopausal. One of these patients had complete remission of pulmonary metastases lasting 12 months and the second patient had no progression of osseous metastases for 12+ months despite the fact that the menstrual cycle was not completely suppressed. Menses were delayed and of shorter duration in both patients. In the first patient, pulmonary metastases recurred after 1 year on tamoxifen. At that time bilateral salpingo-oophorectomy was performed and tamoxifen was continued. Complete regression of pulmonary metastases has again occurred lasting 4+ months. The dose of tamoxifen is being escalated in the second patient to determine whether the menstrual cycle can be completely suppressed. It is apparent that 40 mg/day of tamoxifen is insufficient to completely suppress the menstrual cycle and thus may not yield optimal antitumor effects in premenopausal patients.

TABLE 1.—Results of tamoxifen therapy in 39 patients with stage IV breast cancer

Response	No. of patients (%)	Duration (mos)	No. still in remission
Remission (CR + PR)	19 (49)	11+ (4–18+)	10
No progression	7 (18)	13+ (9–18+)	6
Failure	13 (33)	—	—

Thirty-seven patients were postmenopausal, and they have been divided into three groups according to their postmenopausal status (table 2). There is a relatively high response rate in the 0–5-year postmenopausal group, which may be accounted for by the fact that six of the 12 patients had undergone therapeutic oophorectomy and all six patients had remissions after castration as well as with tamoxifen.

Table 3 presents the relationship between the dominant site of metastasis and the remission rate. In this comparison, only patients who obtained objective tumor regression are considered in relation to those who failed to respond. It is apparent that there was no difference in response rate with visceral, bone, and soft tissue involvement. In this series, visceral involvement consisted primarily of pulmonary metastases, and only one patient had moderately advanced liver metastases.

Table 4 shows the response to previous therapy versus the response to tamoxifen. Twenty patients had received no prior therapy and eight of these patients obtained objective remissions. Of the 11 patients who had responded to prior endocrine therapy, six had obtained remissions from oophorectomy and all six responded to tamoxifen. Other forms of prior endocrine treatment consisted of administration of estrogen, progestin, or androgen. Of five patients who had undergone previous cytotoxic chemotherapy, three obtained objective remissions with tamoxifen.

Table 5 shows the response rate in relation to the presence or absence of estrogen receptors in the metastatic tumors. Four patients who had undetectable estrogen receptors in their tumors failed to respond to tamoxifen. Of 11 patients with positive estrogen receptors, six obtained objective remissions with antiestrogen treatment. These preliminary results suggest that receptor measurements may be useful in selecting patients for this treatment.

Serum prolactin levels have been measured in eight patients who received tamoxifen in order to determine whether antiestrogen therapy might in-

TABLE 2.—Menopausal status and response to tamoxifen in 39 patients

Menopausal status	No. of patients	Response		No progression		Failure	
		No.	%	No.	%	No.	%
Premenopausal	2	1	50	1	50	—	—
Postmenopausal 0–5 yrs	12	7	58	2	17	3	25
Postmenopausal 5–10 yrs	7	3	43	0	—	4	57
Postmenopausal 10+ yrs	18	8	44	4	23	6	33

TABLE 3.—Remissions vs failures in relation to dominant site of disease

Disease site	No. of remissions/ failures	% response
Visceral	6/4	60
Osseous	6/4	60
Soft tissue	7/5	71

fluence the secretion of prolactin. The results in one of the patients are presented in figure 1. Diurnal variation of serum prolactin with a rise during sleep, suppression after L-dopa administration, and stimulation after chlorpromazine administration was similar before and after 9 months on tamoxifen in this patient. Similar results were obtained in seven other patients, indicating that tamoxifen had no significant effect on prolactin secretion.

Five patients who failed to benefit from antiestrogen therapy subsequently underwent hypophysectomy, and one of these five patients obtained an objective remission from hypophysectomy which has lasted > 1 year. Two patients who relapsed after tamoxifen-induced remissions have undergone hypophysectomy, and one of these patients failed to benefit while the other has shown no progression of her disease 6 months after hypophysectomy. Further studies are needed to determine whether hypophysectomy can induce further significant improvement beyond that which can be obtained with antiestrogen therapy.

Table 6 shows the results of tamoxifen therapy in 12 patients who had previously undergone hypophy-

TABLE 4.—Previous treatment and response to tamoxifen

Previous treatment	No. of patients	Remission		No progression		Failure	
		No.	%	No.	%	No.	%
No previous therapy	20	8	40	4	20	8	40
Response to prior endocrine treatment	11	8	73	2	18	1	9
Failure to previous endocrine treatment	3	1	33	1	33	1	33
Prophylactic oophorectomy	3	1	33	—	—	2	66
Previous chemotherapy	5	3	60	—	—	2	40

TABLE 5.—Estrogen receptors* and response to tamoxifen

Estrogen receptor—	No. of patients	Remission		No progression		Failure	
		No.	%	No.	%	No.	%
Positive (> 3 fmols/mg protein)	11	6	58	2	18	3	27
Negative	4	—	—	—	—	4	100

*Estrogen receptors were determined by dextran-coated charcoal method incubating aliquots of cytosol with increasing amounts of labeled estradiol (0.015–0.2 pmol); values were derived from Scatchard analysis.

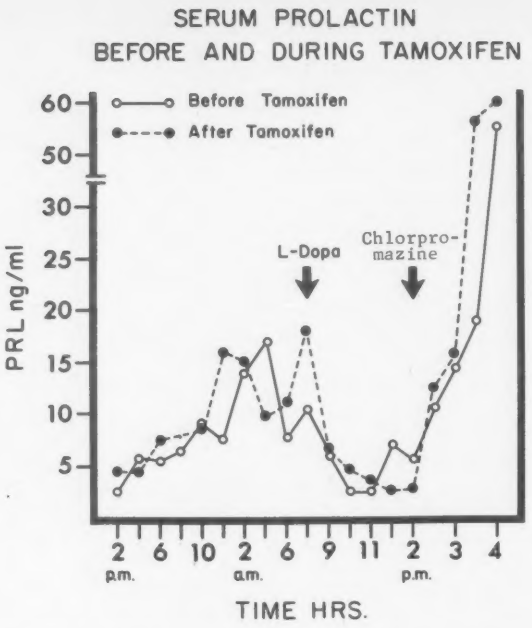

FIGURE 1.—Serum prolactin levels during the day, at night during sleep, after 500 mg of L-dopa orally, and after 50 mg of chlorpromazine im in a patient with metastatic breast cancer. Studies were done before and 9 mos after tamoxifen administration.

sectomy or adrenalectomy. Of nine patients who had previously obtained objective remissions from hypophysectomy, two had objective tumor regression from tamoxifen for periods lasting 4 and 8+ months. Pituitary function studies, including measurements of prolactin, growth hormone, and gonadotropins, indicated that hypophysectomy was complete in these patients. This observation suggests that estrogens may have a direct action in stimulating tumor growth in the absence of the pituitary gland, or possibly tamoxifen may have an antitumor effect which is unrelated to its antiestrogenic activity. One patient who failed to benefit from adrenalectomy obtained objective improvement with tamoxifen which lasted 7 months.

TABLE 7.—Incidence of side effects from tamoxifen in 51 patients

Side effect	No. of patients
Nausea	8
Vomiting	1
Vaginal discharge	7
Vaginal bleeding	2
Hot flushes	9
Distaste for food	3
Pruritus vulvae	1
Skin rash	1
Lightheadedness	1
Depression	1

Table 7 lists the side effects seen in 51 patients treated with tamoxifen. Twenty-six patients had no symptoms while taking the drug. The side effects in the other 25 patients were of minimal severity and usually transitory. In only one patient was the drug withdrawn because of persistent nausea, and this symptom may have been caused by the patient's disease rather than the drug.

DISCUSSION

It is apparent from these studies that tamoxifen is an effective antitumor agent in women with stage IV breast cancer, and the studies confirm the results of other investigators (1–5). The duration of remissions in our series of patients (11+ months) is approaching that obtained with endocrine ablative procedures such as oophorectomy, adrenalectomy, and hypophysectomy (7), and it seems possible that antiestrogens might provide an effective substitute for these surgical procedures. However, further studies are needed to establish this. The remission duration in our series exceeds those obtained with administration of pharmacologic doses of androgens, progestins, and corticosteroids and possibly those of pharmacologic doses of estrogens in postmenopausal women. The side effects of tamoxifen would appear to be less than with pharmacologic doses of steroid hormones, and thus far we have seen no evidence of

TABLE 6.—Results of tamoxifen therapy in 12 patients after hypophysectomy or adrenalectomy

		No. of—		
	No. of patients	Remissions	No progression	Failures
Posthypophysectomy	9	2	3	4
Postadrenalectomy	3	1	—	2

stimulation of tumor growth with tamoxifen administration as may occasionally occur with sex steroid administration. Further studies will be needed to determine whether antiestrogen therapy can replace endocrine ablative therapy for breast cancer.

The results of tamoxifen in our two premenopausal patients are of particular interest. A dose of 40 mg/day of tamoxifen failed to interrupt the menstrual cycle and yet complete regression of pulmonary metastasis occurred in one patient and no progression of osseous metastases was seen in the other. This suggests that a complete block of estrogen action is not necessary to obtain regression of hormone-sensitive cancers. However, further tumor regression was obtained in one of these patients following bilateral oophorectomy, indicating that more complete suppression of estrogen action is needed for optimal antitumor effects. Escalation of the dose of tamoxifen is being tried to determine whether the menstrual cycle can be completely suppressed with larger doses of the drug. Sporadic measurements of serum follicle-stimulating hormone, luteinizing hormone, estradiol, and estrone in these premenopausal patients have shown slightly elevated values for both gonadotropins and estrogens. This suggests that, with the administration of an antiestrogen, the hypothalamic pituitary axis senses a decrease in estrogen activity which results in increased gonadotropin release and, in turn, increased estrogen production by the ovaries. Whether increasing the dose of antiestrogen can overcome the effects of increased estrogen production is uncertain, but the menstrual cycle provides a convenient biologic parameter to determine whether estrogen action has been more completely suppressed. Thus, at present, it is not certain that antiestrogen drugs can substitute for surgical castration in patients with breast cancer.

The results of tamoxifen administration in post-hypophysectomy patients are also of particular interest. Two patients who had previously had objective remissions from hypophysectomy obtained further objective tumor regression with antiestrogens for periods lasting 4 and 8+ months. Both of these patients were studied to determine whether hypophysectomy might have been incomplete. Serum prolactin was undetectable after chlorpromazine administration, serum growth hormone was undetectable after arginine infusion, and serum gonadotropins were at very low levels or undetectable, indicating that hypophysectomy was presumably complete. Serum estradiol and estrone were not measured in these two patients, but in other hypophysectomized patients very low levels of estradiol and estrone have been detected. These observations suggest that small amounts of estrogen may be present in the patient after pituitary ablation and that estrogens may have a direct action in stimulating tumor growth in the absence of the pituitary gland. Another possibility is that tamoxifen may have an antitumor effect which is unrelated to its antiestrogenic activity.

The question as to whether other hormones such as prolactin and growth hormone may play a role in maintaining the growth of human breast cancer is of interest and our studies may provide further information on this problem. Potent antiprolactin drugs have been studied in patients with stage IV breast cancer (8) but, thus far, they have failed to induce objective tumor regression. It is possible that human growth hormone has lactogenic properties and that this hormone may also have to be suppressed to obtain tumor regression. Thus, the effects of hypophysectomy in patients who have had antiestrogen-induced remissions will be of interest. Two patients who relapsed after tamoxifen-induced remissions have undergone hypophysectomy; one of these patients failed to benefit and the other patient has shown no progression of disease after 6 months. Five patients who failed to benefit from antiestrogen therapy subsequently underwent hypophysectomy, and one of these five patients obtained an objective remission which has lasted > 1 year. Further studies are needed to determine whether hypophysectomy can induce further significant improvement beyond that which can be obtained with antiestrogen therapy.

In summary, tamoxifen is an effective antitumor agent in women with stage IV breast cancer. The results obtained to date are approaching those obtained with endocrine ablative therapy. Further studies are needed to determine whether antiestrogens can provide an effective substitute for oophorectomy, adrenalectomy, or hypophysectomy.

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Evaluation of Tamoxifen Dose in Advanced Breast Cancer: A Progress Report

Douglass C. Tormey,[1,2,*] Richard M. Simon,[1] Marc E. Lippman,[1] Joan M. Bull,[1] and Charles E. Myers[1,3]

SUMMARY

The results of an ongoing trial randomizing patients with progressive, metastatic breast carcinoma between tamoxifen (Tam, NSC-180973) and Tam plus fluoxymesterone (Flu) (7 mg/m² bid) are reported. Each patient received a single dose level of Tam in the range of 2–100 mg/m² bid. The combination had a higher response rate overall (45% vs 28%) and when only the patients' soft tissue sites were analyzed (54% vs 9%, $P = 0.04$). The time to treatment failure was longer for the combination among those patients with a response or disease stabilization ($P = 0.08$). Response rates with Tam doses < 12 mg/m² bid were also higher than with doses ≥ 12 mg/m² for all patients in the study (62% vs 30%, $P = 0.025$) and for those where only soft tissue sites were evaluable (43% vs 29%, $P = 0.07$). Side effects were mild and consisted primarily of transient hematologic suppression, nausea, masculinization, hepatic enzyme elevations, and edema. The latter three were observed only with the Flu regimen. Leukopenia and thrombocytopenia were more frequent at Tam doses < 12 mg/m² bid whereas nausea was more common at higher doses. Tam doses as high as 100 mg/m² bid were well tolerated. Tam may be more effective at low doses, has only mild side effects, and is well tolerated at doses up to 100 mg/m² bid. Combining Tam with Flu appears to enhance the therapeutic effectiveness.

[Cancer Treat Rep 60:1451–1459, 1976]

Tamoxifen (Tam), a nonsteroidal antiestrogen, is the *trans* isomer of 1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-but-1-ene citrate. Although the use of Tam clinically was not reported until 1971 (1), the effects of other antiestrogens in patients with breast carcinoma were first reported in 1960 (2). Initial clinical trials suggested that Tam was at least as active as estrogen analogs in the treatment of disseminated breast cancer in postmenopausal women (1,3,4). Response rates between 15% and 60% were reported using dosages of 10 or 20 mg given once or twice daily. The side effects appeared to be equivalent to or less than those of estrogen derivatives; however, little information was available relating wider drug dose ranges to either toxicity or therapeutic effectiveness. The present progress report outlines our initial experience utilizing Tam doses from 2 to 100 mg/m² twice daily with and without simultaneous fluoxymesterone (Flu)[4] administration. The use of Flu was an outgrowth of the observation that some human breast cancers contained androgen-receptor activity in the absence of estrogen-receptor (ER) activity (5,6). This observation suggested that combining the two agents might provide therapeutic effectiveness greater than with either drug alone.

[1]Medicine Branch (Drs. Tormey, Lippman, Bull, and Myers) and Biostatistical Information Systems Unit (Dr. Simon), Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[2]*Present address:* Department of Human Oncology, University Hospitals, University of Wisconsin, Madison.

[3]We wish to thank ICI, United States, Inc, for supplying the tamoxifen, and Dr. Johannes Blom (Walter Reed Army Medical Center, Washington, DC) and Dr. Elliot Perlin (National Naval Medical Center, Bethesda, Md) for their participation.

**Reprint requests to:* Douglass C. Tormey, MD, PhD, Department of Human Oncology, Rm 701C, University Hospitals, Madison, Wis 53706.

[4]Fluoxymesterone: NSC-12165; CAS reg. No. 76–43–7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-.

MATERIALS AND METHODS

Female patients with biopsy-proven evidence of metastatic breast carcinoma and progressive disease were eligible for the trial. Patients excluded from the study included those with organ dysfunction unrelated to tumor, other previous cancers, prior exposure to androgens, a Karnofsky performance index ≤ 30, or a disease manifestation of only mixed osteolytic/osteoblastic disease or pleural effusions. Informed consent was obtained from all patients.

The patients were stratified by dominant disease site (visceral, osseous, or soft tissue), number of organ sites of involvement (≤ 2 vs > 2), measurable disease (yes or no), and prior hormone response (yes, no, or unknown). If prior hormone response was unknown, the patients were stratified by ER status into positive, negative, or unknown. ER was assayed using a competitive protein-binding assay described elsewhere (6). Following stratification, the patients were randomized between Tam and Tam plus Flu using a balanced cell technique (7).

The Flu dosage was 7 mg/m² orally bid. The dose of Tam for the first three entries into the trial was 2 mg/m² orally bid. In the absence of significant acute toxicity, the dosage for every fourth patient was increased sequentially to 4, 8, 16, 32, 50, 60, 80, and 100 mg/m² orally bid. When a dose of 100 mg/m² bid was reached, the dose sequencing was reversed with the aim of providing three evaluable patients on Tam alone at each dose level except 50 mg/m² bid. Just prior to the current analysis (March 1976) the randomization was adjusted to include the Tam dose in addition to the treatment regimen. Each patient entering the trial remained at the entering dose level until disease progression.

Patients were evaluated prior to therapy and at 1–3-month intervals by physical examination, history, complete blood cell counts, liver and renal function tests, chest and skeletal roentgenograms, and bone, liver, and brain scintiscans. During the first 2 months of therapy the patients were evaluated every 1–2 weeks by history, physical examination, blood counts, and liver function tests.

A response (RESP) was defined as at least a 50% reduction in the longest bidimensional perpendicular products, a 30% reduction in unidimensional measurements, or at least a 50% reduction in evaluable disease parameters, eg, lymphangiitic pulmonary disease. For osseous disease partial recalcification was required. The reduction in tumor parameters had to be present in at least 50% of the involved organ sites for at least 28 days to qualify for a RESP. Improvement (IMP) was defined for osseous disease as an objective no change (NC) associated with a marked reduction in analgesic requirements or a 20-point improvement in the Karnofsky performance index when these subjective indices were associated with osseous disease. The three patients meeting this criterion are classified under RESP in the Results section since their time to treatment failure was similar to that of other responding patients. Those patients not achieving RESP, IMP, or progressive disease (PD) within 8 weeks of starting therapy were classified as NC. PD was defined as the appearance of any new lesion or a 25% increase in the disease measurements of the lesions in any organ site as long as the increase was at least 2 cm² for bidirectionally or 2 cm for unidirectionally measured lesions and persisted for 7 days. A relapse (REL) was defined as PD occurring after NC, IMP, or RESP status; the minimal measurements obtained were utilized as the baseline for comparison.

Statistical analyses were performed using the Fisher Exact Test, the Generalized Wilcoxon Test, or the Wilcoxon Rank Test as appropriate. For combined comparisons among contingency tables, the Mantel-Haenszel Test was used. All P values correspond to one-tailed tests. Toxicity criteria are detailed in the text.

RESULTS

Patient Characteristics

Forty-five patients have thus far been entered in the study. One patient was ineligible because of a low performance status. One patient with nonmeasurable disease was eligible for toxicity analysis but not for the response analysis. Two patients were lost to followup during the first week of therapy and it is too early for analysis in three patients. Thus, there are presently 18 patients who received Tam alone and 20 patients who received the combination who are evaluable for response analysis. The one additional patient evaluable for toxicity analysis received the combination regimen with a Tam dose of 12 mg/m² bid.

Forty-seven percent of the patients evaluable for response had visceral dominant, 37% had osseous dominant, and 16% had soft tissue dominant disease. Among the 26 patients who had undergone previous hormone therapy, 13 (50%) had responded, 11 (42%) had not responded, and two were not evaluable. ER data were available for 21 patients. Eighteen (86%) of these were ER positive (> 3 fmols/mg cytoplasmic protein) and three were ER negative. In four of the ER-positive cases the ER analysis was performed after REL from either NC (three cases) or RESP (one case) to protocol therapy.

The patients had received an average of 1.5 chemotherapy regimens each and 37 of the 38 patients had received prior chemotherapy. Four patients were premenopausal and half of the remaining 34 postmenopausal patients had undergone prior oophorectomy.

The population had a median Karnofsky performance status of 80 (range, 35–100), age of 50 years (range, 24–67), disease-free interval of 1.4 years (range, 0–14), time from mastectomy or diagnosis of 2.8 years (range, 0.25–19), and two organ sites (range, 1–5) of disease involvement.

Thus, the population was characterized by advanced, previously treated metastatic disease. There was no major difference between the groups receiving the two regimens with respect to these characteristics.

Response to Therapy

The response to therapy is depicted in table 1 as a function of both the regimens and the Tam dosage. The overall response rate is 28% for Tam (five of 18 patients) and 45% for the combination of Tam and Flu (nine of 20 patients) ($P > 0.10$). The response rate at Tam doses < 12 mg/m² bid is higher in both regimens, and in the combined data from both regimens is 62% (five of eight patients) as compared to 30% (nine of 30 patients) at doses ≥ 12 mg/m² bid ($P = 0.025$, Mantel-Haenszel Test). Among the patient characteristics there was an imbalance of prior hormone responders in the < 12-mg/m² bid group. Consideration of this imbalance resulted in corrected P values for the dose effect of 0.15 for Tam alone and 0.065 for Tam with or without Flu. Thus, it appeared that the response rate was higher at Tam doses < 12 mg/m² bid.

Since soft tissue sites of disease are classically more sensitive to hormonal therapies than visceral or osseous sites of disease, the patients with soft tissue sites of involvement were re-analyzed with respect to those sites only. The results of this analysis are shown in table 2. Again, the response rate is higher in those patients receiving < 12 mg/m² bid of Tam ($P = 0.07$). This was not changed by considering pretherapeutic variables. In addition, the response rate is higher (54% vs 9%) in those patients receiving the combination of Tam and Flu ($P = 0.04$). Consideration of the pretherapeutic variable of prior hormone response resulted in correction of the P value for the combination's superiority to 0.015. Thus, the response rates in this analysis appear to be superior for the combination regimen and again for doses < 12 mg/m² bid.

Because of the difference in dosage effects, those patients in whom the drug dose was altered following REL were further evaluated. One patient relapsing after NC on 4 mg/m² bid of Tam continued to develop PD when the dose was increased to 50 mg/m² bid. Another patient relapsing after NC on 32 mg/m² bid of Tam continued to have PD when the dose was reduced to 2.5 mg/m² bid. The course of a third patient who received the combination is shown in figure 1. After relapsing from RESP while on 3.75 mg bid, the dose was increased to 50 mg bid while the Flu was continued at 7 mg/m² bid. A transient

TABLE 1.—Response to therapy related to regimen and dose of Tam

Regimen	Tam dose (mg/m² bid)	No. of— RESP (%)	NC	PD	P value
Tam	< 12	2 (50)	2	0	0.06
	≥ 12	3 (21)	4	7	
Tam + Flu	< 12	3 (75)	0	1	0.16
	≥ 12	6 (38)	2	8	
Total	< 12	5 (62)	2	1	0.025
	≥ 12	9 (30)	6	15	

TABLE 2.—Response to therapy (soft tissue sites only) of those patients with such sites of involvement

Tam dose (mg/m² bid)	Tam			Tam + Flu			Total		
	RESP (%)	NC	PD	RESP (%)	NC	PD	RESP (%)	NC	PD
< 12	0	3	0	3	1	0	3 (43)	4	0
≥ 12	1	3	4	4	2	3	5 (29)	5	7
	1 (9)	6	4	7 (54)	3	3	$P = 0.07$		
	$P = 0.04$								

TABLE 3.—Effect of pretherapy variables on the response rate to Tam ± Flu

Characteristic	No. of— RESP (%)	NC	PD	*P* value
Dominant disease				
Visceral	4 (22)	3	11	0.032
Osseous	6 (43)	5	3	0.085
Soft tissue	4 (67)	0	2	
Disease-free interval				
< 2 yrs	6 (26)	5	12	0.03
≥ 2 yrs	8 (57)	3	3	
Prior hormone therapy				
Response	7 (54)	2	4	0.017
No response	2 (17)	2	8	

decrease in disease was followed by continued PD and the drugs were discontinued. The patient then underwent a drug-withdrawal RESP lasting 4+ months. Upon REL from this RESP the patient failed to respond to 3 mg bid of Tam. No other withdrawal RESPs were recorded in the 38 patients. These observations suggest that withdrawal RESPs are unusual and that altering the Tam dose level during PD is unlikely to "rescue" a RESP.

The effect of those pretherapy variables which appeared to influence the response rate to Tam with or without Flu are shown in table 3. The response rate with visceral dominant disease (22%) was lower than with either osseous (43%) or soft tissue (67%) dominant disease. The response rate with disease-free intervals ≥ 2 years (57%) was higher than with disease-free intervals < 2 years (26%). Similarly, the response rate in patients who had responded to prior hormonal therapy (54%) was higher than in those patients not responding to prior hormonal therapy (17%). With respect to prior hormone therapy, five of eight prior oophorectomy responders also responded to Tam with or without Flu as compared to one of five prior oophorectomy failures. One patient responding to prior oophorectomy plus adrenalectomy failed to respond. A prior estrogen RESP was associated with RESP to Tam with or without Flu in two of four patients as compared to one of seven prior estrogen nonresponders. There were six of 18 (30%) responders among ER-positive patients compared to one of three in ER-negative patients. These RESPs occurred in two of ten patients receiving Tam alone (none of two at < 12 mg/m²; two of eight at ≥ 12 mg/m²) and in four of eight receiving the combination (two of two at < 12 mg/m²; two of six at ≥ 12 mg/m²). Among premenopausal patients there were two PDs (50 and 60 mg/m² bid, Tam alone), one RESP (60 mg/m² bid, Tam plus Flu), and one NC (32 mg/m² bid, Tam alone). Among postmenopausal patients there were seven RESPs,

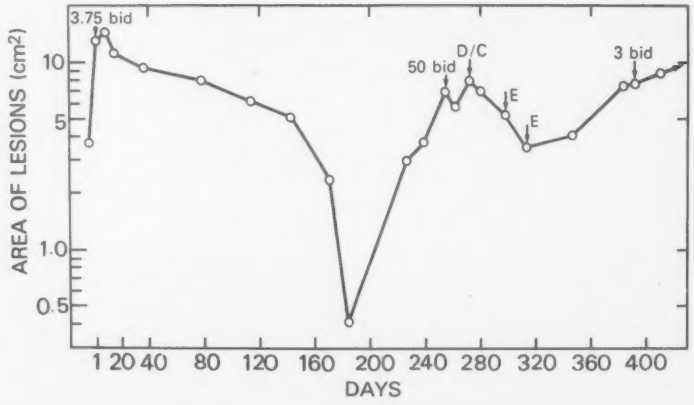

FIGURE 1.—Course of a patient receiving Tam and Flu. Area of the skin lesions is plotted against time from starting therapy. Dosage changes of Tam are indicated. D/C = drugs discontinued. ↓E = decreased erythema of lesions. Patient's surface area = 1.5 m².

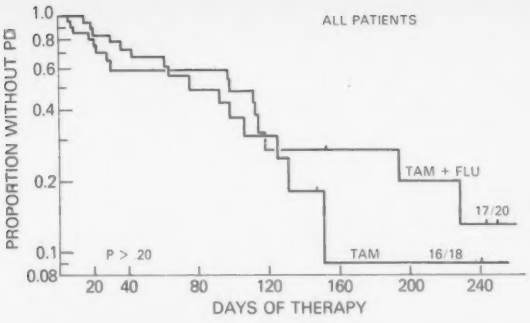

FIGURE 2.—Time to therapeutic failure for all patients entered in the 2 regimens. Fractions indicate number of patients who have relapsed or progressed over the total number of patients in each life-table plot.

five NCs, and five PDs among prior surgical castrates, and six RESPs, two NCs, and nine PDs among patients naturally postmenopausal. Three patients relapsing after RESP or NC to Tam with or without Flu underwent adrenalectomies; one patient had RESP, one had NC for 4+ months, and one developed further PD within 1 month. Thus, those features usually associated with higher response rates to hormonal therapies are also manifested in this trial, ie, soft tissue dominant disease, prior hormone responsiveness, and longer disease-free intervals.

There were no differences in the time from initiation of therapy to therapeutic failure between the two regimens for all patients (fig 2) or for responders. However, the combination was slightly

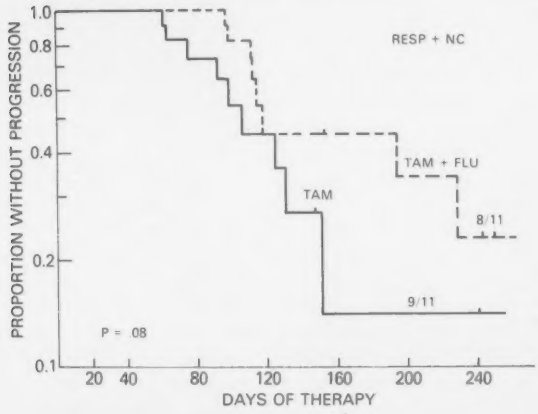

FIGURE 3.—Time to therapeutic failure for patients achieving RESP or NC on the 2 regimens. Fractions indicate the number of patients who have relapsed over the total number of patients in each life-table plot.

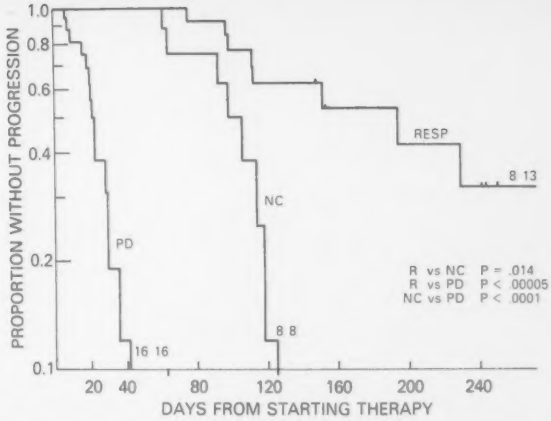

FIGURE 4.—Time to therapeutic failure for patients with RESP, NC, or PD to Tam with or without Flu. Fractions indicate the number of patients who have progressed or relapsed over the total number of patients in each life-table plot.

superior when considering those patients with RESP or NC (fig 3). The median time to initiation of RESP in the 14 responders was 37 days. The median time to treatment failure was 6.4 months for responders, 3.2 months for patients achieving NC status, and 22 days for patients with PD (fig 4). Thus, disease control appears to be superior for the combination among patients with RESP or NC, and is superior for responders as compared to patients with NC or PD.

Toxicity

Hematologic side effects appeared to be similar between the two regimens (table 4). Patients at all dose ranges had hemoglobin decreases > 1 g/100 ml. There was a trend for more leukopenia (< 4000 cells/mm^3) or thrombocytopenia (< 100,000 cells/mm^3) at Tam doses < 12 mg/m^2 bid as compared to higher doses (57% vs 35% and 25% vs 8% respectively). There were no differences between 12–60 and 80–100 mg/m^2 bid among these parameters. No patients had leukocyte counts < 2000 cells/mm^3 or platelet counts < 75,000 cells/mm^3.

The median time for hemoglobin decreases > 1 g/100 ml was Day 14 with return to previous levels 7 days later. Similarly, the median time of leukopenia was Day 13 with return to > 4000 cells/mm^3 12 days later. Thrombocytopenia occurred within those same time intervals. In most cases the drugs were continued despite leukopenia or thrombocytopenia. In retrospect, there was no patient in which the peripheral counts necessitated interruption of therapy.

TABLE 4.—Hematologic side effects

Parameter	Tam dose (mg/m^2 bid)	No. of patients with decrease/No. of evaluable patients (%)		
		Total	Tam	Tam + Flu
Hgb decrease > 1 g/100 ml	< 12	1/8 (12)	1/4	0/4
	≥ 12	7/28 (25)	4/14	3/14
Wbc count < 4000 cells/mm^3	< 12	4/7 (57)	1/3	3/4
	≥ 12	8/23 (35)	5/12	3/11
Platelet count < 100,000 cells/mm^3	< 12	2/8 (25)	1/4	1/4
	≥ 12	2/26 (8)	2/13	0/13

TABLE 5.—Nonhematologic side effects

Side effect	No. of patients with effect/No. of evaluable patients	
	Tam	Tam + Flu
Gastrointestinal	4/18	2/21
Hepatic	0/18	3/21
Masculinization	0/18	4/21
Edema	0/18	5/21
Skin rash	1/18	0/21
Infection	2/18	0/21
Central nervous system	0/18	1/21
"Flare"	3/18	3/21

The incidence of nonhematologic side effects is shown in table 5. Gastroenteric side effects occurred in none of eight patients at Tam doses < 12 mg/m^2 bid as compared to five of 12 patients at doses of 12–60 mg/m^2 bid and one of ten patients at doses of 80–100 mg/m^2 bid. One patient receiving Tam alone had multiple emesis during one 24-hour period and one patient receiving the combination had emesis associated with acute pancreatitis which was not considered to be drug related. The remainder of the gastroenteric side effects consisted of mild and transient nausea only.

Elevation of hepatic enzymes, masculinization, and/or edema occurred in eight of 21 patients receiving the combination as compared to none of 18 patients receiving Tam alone ($P = 0.003$). One patient receiving 100 mg/m^2 bid of Tam alone developed a diffuse erythematous skin rash necessitating the cessation of therapy. One patient receiving Tam alone developed a urinary tract infection and another patient had an upper respiratory infection. Signs and symptoms of cerebellar dysfunction developed in one patient receiving the combination. This disappeared after several weeks despite continuing therapy and was not associated with demonstrable pathology.

A disease "flare" or presumed disease exacerba-

TABLE 6.—Characteristics of the clinical "flare" in 8 patients

Regimen	Tam dose (mg/m^2 bid)	Response	Type of flare	No. of days—	
				Flare was observed	From start of therapy to treatment failure
Tam	2	IMP	Increase in osseous pain	5–33	75
	4	NC	New skin lesion; increase in osseous pain	14–48	92
	8	RESP	New extradural lesion	3–33	131
	32	NC	New skin lesion; increase in other skin lesions with erythema	13–27	125
Tam + Flu	2	RESP	Increase in skin lesion and erythema; increase in osseous pain	7–36	194
	8	RESP	Increase in skin lesion	7–28	229
	12	PD	Increase in skin lesion; new node lesion	2–20	6
	50	RESP	Increase in skin lesion	1–22	112

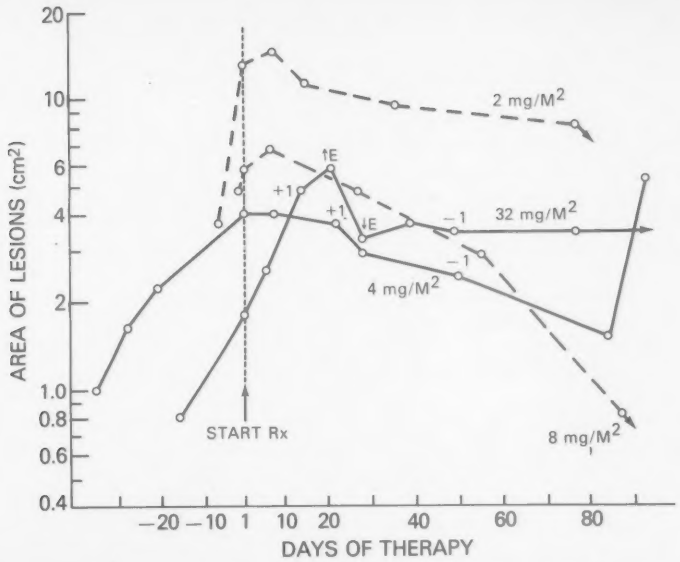

FIGURE 5.—Course of patients considered to have the characteristics of a "flare." Sum of the area of the skin lesions is plotted against the days of therapy. The bid dose of Tam for each patient is indicated. Solid lines = Tam alone; broken lines = Tam and Flu; E = erythema of lesions; +1 and −1 = appearance and disappearance of new lesions.

tion was recorded in the clinic in three patients on each regimen and similar symptomatology was identified in two additional patients (table 6). These reactions occurred at Tam doses from 2 to 50 mg/m² bid and were associated with a subsequent RESP in five cases, NC in two cases, and PD in one case. The reactions began within 2 weeks of initiating therapy, began to subside after 1–2 weeks, and returned to at least pretherapy levels within 5 weeks. The soft tissue reactions consisted of the development of new lesions and/or an increase in the size of preexisting lesions sometimes associated with marked erythema within and around the lesions. In patients with osseous disease the reactions were associated with a marked increase in pain manifested by increased analgesic requirements. In one patient there was a marked increase in pain associated with the development of a cauda equina compression syndrome. Therapy was interrupted and the lesion responded to drug withdrawal and radiation therapy, after which hormonal therapy was started again with the subsequent development of a RESP in other lesions. In no case was hypercalcemia encountered.

That the "flare" was only a manifestation of preexisting rapid tumor growth is suggested by the clinical growth curves of four patients with soft tissue lesions (fig 5). In three cases the growth curves began to attenuate within 8 days after initiating therapy. The fourth patient's lesions continued to increase for 2 weeks before attenuation began.

DISCUSSION

The present report further confirms the results of Cole et al (1,4) and Ward (3) indicating that Tam is an active antitumor compound in patients with advanced breast cancer. In addition, the combination of Tam with Flu appeared to be associated with higher response rates and longer durations of disease control among those with RESP or stabilization compared to Tam alone.

The results thus far reported also suggest that Tam will be at least as active as estrogens such as diethylstilbestrol; however, definitive clinical trials comparing the two have not yet matured. It is of interest, however, that another antiestrogenic compound, 2α,3α-epithio-5α-androstan-17β-ol, was found to cause regressions in 12 of 42 (29%) patients when compared double-blind to testosterone propionate which caused regressions in seven of 40 (18%) patients (8). A third antiestrogenic compound, nafoxidine, also appears to have antitumor activity similar to that of Tam and 2α,3α-epithio-5α-androstan-17β-ol. Nafoxidine has been reported to produce regressions in from 35% (eight of 23) to 37% (18 of 43) of the patients treated (9,10). Thus, the antiestrogens

appear to be an active group of agents for the treatment of advanced breast cancer.

The sequential Tam trials of Ward were associated with response rates of 51% at a dose of 10 mg bid and 60% at a dose of 20 mg bid (3). Both these dosages are close to the < 12-mg/m² bid dose that appears to be superior to higher doses in our ongoing trial. There is no information currently available to explain why the drug should be more active at lower doses. It is conceivable, however, that higher doses may be estrogenic as has been demonstrated in the rat (11). High doses may also lead to interaction with other receptors such as androgen receptors.[5] Alternatively, metabolites of the parent drug (12,13) may provide these interactions at higher doses.

The apparent enhanced therapeutic effectiveness when Flu is given simultaneously with Tam may be due to an additive effect of Flu interacting with androgen receptors in cells devoid of or containing biologically ineffective ER activity. This hypothesis is strengthened by the observation by us and others that some breast cancer tissues contain dihydrotestosterone-receptor activity in the absence of ER activity (5,6). Alternatively, the Flu could be acting through feedback loops, for example at the pituitary.

Finally, the side effects observed in the present study were similar to those reported by others for Tam (1,3,4) and Flu (14). The most frequent side effects noted were hematologic suppression, nausea, edema, masculinization, and hepatic enzyme elevations. The latter three appeared to be related to the Flu because they did not occur in the Tam-alone regimen. Nausea and peripheral blood count suppressions were mild and transient and apparently rarely need interfere with continuation of therapy. There was a suggestion that leukopenia and thrombocytopenia were more frequent at lower dose levels and that the gastroenteric side effects were more frequent at higher dose levels. There was no clear relationship of other side effects to the Tam dosage and 100 mg/m² bid was well tolerated. Tam was not associated with the severe skin photosensitivity reported with nafoxidine (9,10) or the mild androgenic side effects reported with 2α,3α-epithio-5α-androstan-17β-ol (8).

The clinical observation described as a "flare" of the disease was defined as increased erythema and/or size of skin lesions, appearance of new soft tissue lesions, and/or increased pain associated with osseous lesions. These changes began within 2 weeks of initiating therapy and at first were thought to represent stimulation of the growth of the tumor. However, plotting the growth characteristics of the lesions prior to and during therapy suggested that the size changes of the lesions were continued growth of the disease for up to 3 weeks after starting therapy. There was no indication that the growth curves had accelerated. The bone pain and lesion erythema are best explained by an infiltration of reactive cells and/or the development of a drug-induced unbalanced growth state resulting in a temporary increase in the volume of the cells prior to cell death. Biopsy of such skin lesions prior to and during therapy should help classify this reaction. Clark et al have shown that the antiestrogen nafoxidine can stimulate immature uterus before antiestrogenic activity is seen (15). These observations make it very difficult during the first few weeks of therapy to decide whether the patient's disease is progressing or whether it will stabilize or enter remission with continued treatment. The observation that the growth curve has begun to taper by 3 weeks of treatment in those patients whose disease will stabilize or respond suggests that treatment should not be abandoned until at least 3 weeks of careful monitoring has occurred.

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Preliminary Report of a Controlled Trial in Advanced Breast Cancer Comparing Tamoxifen With Conventional Hormone Therapy

M. Maureen Roberts,* A. P. M. Forrest, T. Hamilton, A. O. Langlands, W. Lutz, Ida J. McFadyen, and Helen J. Stewart[1]

Previous reports by Cole et al (1) and Ward (2) indicated that benefit from tamoxifen (NSC-180973) therapy may occur in 30%–40% of women with advanced carcinoma of the breast. The recorded incidence of side effects from the drug was low in both series. In order to determine whether tamoxifen has any advantage over conventional hormone therapy in the treatment of breast cancer, a controlled clinical trial was set up in Edinburgh, with the support of ICI Pharmaceuticals, UK.

The original aims of the trial were to compare tamoxifen with standard estrogen therapy in postmenopausal women and with standard androgen therapy in menopausal patients. Due to the small intake in the latter group, this part of the trial has been discontinued. The current objective of the trial is to compare tamoxifen with diethylstilbestrol[2] in postmenopausal women with proven advanced carcinoma of the breast.

All patients with proven advanced disease attending our combined breast clinic are eligible for the trial, providing the following criteria are met: (*a*) last menstrual period > 5 years previously; (*b*) < 80 years of age; (*c*) no previous hormonal, endocrine, or cytotoxic therapy for advanced disease. Patients who fulfill these criteria are excluded from the study however if they (*a*) have received previous hysterectomy and/or incidental oophorectomy and are < 60 years of age; (*b*) have previous or concurrent carcinoma of another site; or (*c*) are in poor general medical condition contraindicating either of the therapies under study.

Stratification

Patients accepted for the trial are stratified by disease-free interval and site of recurrence. The free interval is divided into three groups (0–1 years, 1–5 years, or > 5 years); disease site is subdivided into localized (limited to the breast, draining lymph nodes, and chest wall) and generalized (distant spread). Stratification therefore gives six subgroups of postmenopausal patients for eventual analysis.

Allocation to treatment within the stratification groups is by a system of random cards prepared by the statistician.

Treatment

Patients receive either tamoxifen at a dose of 10 mg three times a day or diethylstilbestrol at a dose of 5 mg three times a day; the drugs are presented in a similar way. Neither the patient nor the doctor knows the code of the drug, which may be broken only for medical reasons during the trial.

Conduct of the Trial

On entry, each patient has a full clinical examination with measurement and/or photography of all visible and palpable lesions; a full radiologic survey and bone scintiscans are performed. Biochemical tests of liver function, serum calcium, and hematologic indices (including platelet count) are performed and repeated monthly.

The patient is clinically assessed at regular intervals and roentgenograms are repeated at 3 months unless symptoms indicate otherwise.

Response to therapy is assessed according to the criteria suggested by the British Breast Group (3). Response is defined as (*a*) *regression* (objective response)—improvement of all visible, palpable, and radiologic lesions persisting for ≥ 6 months with no new lesions and no other therapy being given; (*b*) *intermediate* response (equivocal)—either *temporary* (< 6 months), *static* (no lesions have progressed but all or most lesions have remained stationary), or *mixed* (some lesions have improved and others have worsened but no new lesions have appeared); and (*c*) *progression* (failure, no response)—all or some lesions have worsened, none have regressed, or new lesions have appeared.

Results are also assessed by two external assessors from another center. Two clinicians with no knowledge of the patient are given access to the

[1]Departments of Clinical Surgery, Radiotherapy, and Medical Statistics, University of Edinburgh, Scotland.

[2]Diethylstilbestrol: NSC-3070; 4,4'-stilbenediol, α,α'-diethyl-.

**Reprint requests to:* Dr. M. Maureen Roberts, Department of Clinical Surgery, The Royal Infirmary, University of Edinburgh, Edinburgh EH3 9YW, Scotland.

notes and roentgenograms and are asked to assess independently the response of the disease to therapy.

Once a patient is judged to have failed to respond to treatment or when a remission is ended, a period of 4 weeks is allowed to elapse, if the patient's general medical condition permits. If no withdrawal response occurs, the patient will then receive the alternative drug therapy and is assessed as described above.

Analysis of the Trial

The trial is a paired sequential crossover trial. Analysis will be done of the incidence of response to each therapy with first and second drug analyzed separately, the mean duration of response to each therapy, and the incidence of morbidity.

Results

The trial is still in progress and to date, 54 patients in the postmenopausal group have been entered. Only 29 patients have completed therapy and been fully assessed. This assessment included external review by two outside clinicians. The drug code for patients within the trial has not yet been broken, since some patients are still on their second (crossover) drug. The overall response rate (including objective and intermediate responses) in patients who received an adequate trial of either drug is 24%. Details are given in table 1.

Full assessment of morbidity cannot be made until the drug code is broken. However, from studies of patients treated electively by tamoxifen in our clinic, we agree with the reported findings of the low incidence of major side effects with this drug.

Estrogen Receptor Assay

Biopsy of accessible tumor tissue is performed before starting therapy within the trial for estrogen receptor protein estimation, by the method previously published by Hawkins et al (4). The validity of estrogen receptor concentration in predicting response to tamoxifen and diethylstilbestrol therapy will be determined.

Comment

This preliminary communication describes the purpose and structure of a randomized trial which is still in progress. It is too soon to discuss its results, but we believe that this trial will give a definitive answer to the question of the comparison of tamoxifen and diethylstilbestrol as a first treatment in postmenopausal patients with advanced breast cancer.

TABLE 1.—Results in 29 fully assessed postmenopausal patients who have received either tamoxifen or diethylstilbestrol as initial therapy

Response	No. of patients
Objective	4
Intermediate	3
None	17
Intolerant of drug	5
	29

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Current Overview of EORTC Clinical Trials With Tamoxifen[1,2]

Jean-Claude Heuson*

SUMMARY

Based upon concepts derived from estrogen receptor studies, a phase II exploratory trial was conducted where tamoxifen (NSC-180973) was administered concomitantly with cytotoxic chemotherapy in postmenopausal patients with advanced breast cancer. Chemotherapy was composed of two 28-day cycles given alternatively. Cycle A consisted of adriamycin and vincristine, and cycle B consisted of cyclophosphamide, methotrexate, and 5-fluorouracil. Fifty-five patients were fully evaluable. Complete remissions were obtained in 13 (24%) and partial objective remissions in 27 (49%). The overall remission rate was 73%. Toxic side effects ranged from mild to severe. The most significant were nausea and vomiting (most patients), weakness and pain (two thirds of the patients), and hematologic changes (half of the patients). It is concluded that the present combination of endocrine and cytotoxic therapy represents one of the most effective currently available treatments of advanced breast cancer. In an early clinical trial of tamoxifen in premenopausal patients with advanced breast cancer, two objective remissions, lasting 5 and 9+ months respectively, were obtained in ten patients treated. Tamoxifen is worthy of further assessment in premenopausal patients.

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This paper reports the preliminary results of two on-going trials using tamoxifen in the treatment of advanced breast cancer. One is based on the concomitant administration of tamoxifen and alternating cyclic chemotherapy to postmenopausal patients. The other investigates the putative therapeutic activity of tamoxifen in premenopausal patients.

In the first trial, the rationale for combining a hormonally active substance with cytotoxic chemotherapy was based on concepts derived from estrogen receptor (ER) studies in breast cancer.

The initial report by Jensen et al (1) on ER in relation to response to endocrine treatments virtually equated presence of ER in breast cancer tissue with its hormone dependence. There appeared to be two distinct categories of patients recognizable by the presence or absence of ER in their tumors who were candidates for either endocrine or cytotoxic treatments. Subsequent studies revealed that this view was an oversimplification and that the relationship between presence of ER and response to endocrine therapy was much looser than initially thought. A reasonable estimate of the predictive value of ER was obtained at a meeting held in Bethesda, Maryland, in July 1974. By pooling the results from eight institutions, it was found that among 436 trials, about half the cases were ER positive (ER^+), half were ER negative (ER^-), and a

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[2]This study was done by the European Organization for Research on Treatment of Cancer (EORTC) Breast Cancer Cooperative Group: chairman, J. C. Heuson; secretary, W. H. Mattheiem; statistician, R. Sylvester; EORTC coordinator, M. Staquet; and EORTC representative, H. J. Tagnon. The following participated in this study: Institut Jules Bordet, Brussels, Belgium (J. C. Heuson); Institut Médical des Mutualités Socialistes, La Hestre, Belgium (J. Michel); Kliniek Minnewater, Brugge, Belgium (A. Clarysse); Centre Henri Becquerel, Rouen, France (J. P. Julien); Antoni van Leeuwenhoek Ziekenhuis, Amsterdam, The Netherlands (E. Engelsman); Rotterdamsch Radiotherapeutisch Instituut, Rotterdam, The Netherlands (D. Blonk-van der Wijst); Finseninstitutet, Copenhagen, Denmark (H. Mouridsen); Universitäts-Krankenhaus-Eppendorf, Hamburg, West Germany (H. Maass); Universitätskliniken, Ulm, West Germany (W. Schreml).

*Reprint requests to: Dr. J. C. Heuson, Service de Médecine et Laboratoires d'Investigation Clinique, Institut Jules Bordet, Rue Héger-Bordet 1, 1000 Brussels, Belgium.

few were "borderline." Fifty-six percent of the ER^+ cases and only 10% of the ER^- cases had objective remissions from endocrine treatments (2). The definition of ER positivity differed however among institutions. Some considered as positive only tumors having ER concentration above a critical level (3). In fact, sensitive assay methods such as the dextran-coated charcoal procedure are disclosing the presence of ER in nearly 80% of breast cancers which renders useless the mere distinction between ER^+ and ER^- tumors (4). Furthermore, the distribution of ER concentrations in positive tumors is continuous from very large to near zero values and there is no indication of the existence of two distinct populations of patients in this regard. This leads to the concept that breast cancers should no longer be regarded as hormone dependent or independent but rather as having varying degrees of dependence that might tentatively be estimated by their levels of ER (4).

This interpretation was tested in a clinical experiment involving 34 treatment trials in 33 patients at our institution. Eight objective remissions were obtained by various endocrine treatments of established efficacy in 25 ER^+ trials (32%) and no remissions were obtained in nine ER^- trials. The ER^+ trials were analyzed for a possible correlation between ER concentration and probability of occurrence of a remission. It was found by using the linear logistic regression model (5) that such a correlation existed and was significant at the 0.02 level. Although this reflected a significantly higher remission rate in receptor-rich tumors, remissions in the present series were associated with probabilities of response estimated from the model ranging from 12% to 77% (6). This finding is consistent with the interpretation tested, according to which there is a continuous gradient of hormone dependency in the ER^+ patients that could even be extrapolated to the so-called negative ones. Although only patients with the higher dependency will show objective remission, that is, recognizable tumor regression after endocrine treatment, the others would derive definite yet clinically unrecognizable benefit. From this viewpoint, endocrine treatment should be an obligatory component of the therapy in most if not all of the patients. It should be complemented with chemotherapy even in cases of high hormone dependency since it is accepted that endocrine treatments have seldom if ever achieved definitive cure of advanced breast cancer. It is conceivable that a combination of both forms of treatment under conditions still to be defined might be more successful in this regard. Verification of this theory was the purpose of the tamoxifen chemotherapy trial.

The second trial was devised to test the possible efficacy of tamoxifen in premenopausal patients. This trial appears especially interesting with regard to extending to this category of patients the combined endocrine-cytotoxic approach currently under investigation in postmenopausal patients.

Combined Tamoxifen-Alternating Cyclic Chemotherapy[3]

Tamoxifen is a typical antiestrogenic compound which, like nafoxidine (7,8), was shown to induce remissions in postmenopausal patients with advanced breast cancer (9,10). It was given orally at a dose of 20 mg twice daily.

Chemotherapy given concomitantly involved an alternation of two 28-day cycles. Cycle A (ADM-VCR) was composed of adriamycin and vincristine given at doses of 75 mg/m^2 iv on Day 1 and 1.4 mg/m^2 iv on Days 1 and 8 respectively.[4] Cycle B (CMF) involved cyclophosphamide, 100 mg/m^2 orally from Days 1 to 14; methotrexate, 60 mg/m^2 iv on Days 1 and 8; and 5-fluorouracil, 600 mg/m^2 iv on Days 1 and 8.[5] Patients were randomly allocated to an alternating schedule: ABAB . . . or BABA. . . . After 12 cycles, that is, after about 1 year, the duration of each cycle was extended to 56 days. Doses were reduced by one third in patients above the age of 68. A dose-reduction program was defined in cases of toxic reactions.

The selection of treatments A and B was based upon their demonstrated efficacy, with rates of objective remissions (CR + PR) of nearly 50%, and the absence of major cross-resistance (11). The rationale for alternating their administration was twofold. On one hand, alternating therapy was based on the assumption that relapse after remission was due to resistance to a given treatment and that perhaps failure to obtain a remission was due to early resistance in such a manner that reduction of tumor size could never be clinically recognized. By starting a second treatment at the time of either recurrence or recognition of failure, the problem of resistance may be overcome but the tumors will have returned to their original size or have grown. If one supposes that alternating treatments in a schedule such as

[3]This study will be reported in full detail in a separate paper by E. Engelsman et al.

[4]Adriamycin: NSC-123127; CAS reg. No. 23214–92–8.

Vincristine: NSC-67574; CAS reg. No. 2068–78–2; leurocristine, sulfate (1:1).

[5]Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

5-Fluorouracil: NSC-19893; CAS reg. No. 51–21–8.

ABAB ... avoids or delays resistance, then each cycle will act on tumors of scalewise decreasing volume until regression is clinically recognized. On the basis of these theoretical assumptions, one may hope to obtain a higher rate and a longer duration of remission.

On the other hand, alternating cycles A and B may hopefully have the advantage of decreasing the cumulative toxicity of drugs such as adriamycin or vincristine thereby delaying the occurrence of toxicity and increasing the maximum tolerated total doses.

Patients were admitted into the study when they had advanced breast cancer and were at least 1 year postmenopausal. They had to meet the criteria of eligibility of the EORTC Breast Cancer Cooperative Group (8). Patients with massive liver involvement were nevertheless accepted provided their performance status was ≥ 60 (Karnofsky index). None had received prior endocrine or cytotoxic treatments.

The experiment was designed as a phase II trial and the primary endpoints were to estimate the rate of objective remission and the duration of remission and survival, and to assess the toxicity of the treatments given.

The criteria of objective remission are those of the EORTC (8). Assessment is performed 4 months after initiation of therapy. One important provision is that all cases are subjected to extramural review by two independent investigators.

To date, 55 patients are fully evaluable. The results are given in table 1. The overall rate of CR is 24% (13 of 55), and the rate of CR + PR is 73% (40 of 55). There was no difference between schedules ABAB ... and BABA .... It is too early to assess duration of response and survival.

The toxic side effects ranged from mild to severe. There were two toxic deaths from leukopenia and infection, one due to accidental overdosage. The most significant side effects were nausea and vomiting, which occurred to some extent in most patients, weakness and pain, of which two thirds of the patients complained, and hematologic changes, which were recorded in half of the patients. Alopecia occurred in all cases.

Tamoxifen Trial in Premenopausal Patients[6]

This phase II trial was conceived with the purpose of merely establishing whether or not a typical antiestrogen was active in at least 20% of premenopausal patients. In this type of trial treatment should be stopped if there are no objective remissions in the first 14 patients.

Tamoxifen was given orally at a dose of 20 mg twice daily. Assessment of response was made at 3 months according to the criteria already quoted of the EORTC (8). Treatment was continued unless there was definite evidence of failure.

Ten patients were fully evaluable and two objective remissions were recorded. One consisted of complete disappearance of ascites and massive omental metastatic involvement, verified at laparotomy; it lasted 5 months, after which time myelophtisic anemia developed. The other remission consisted of complete disappearance of a bulky left supraclavicular lymph node and decrease in size of bilateral hilar masses; it is still continuing 9 months after therapy.

Side effects were negligible, with only some mild nausea and vomiting and occasional hot flushes. Hematology studies were performed at monthly intervals and failed to disclose significant changes. In particular, there was no thrombocytopenia. Effects on menstrual cycles are shown in table 2. Eight patients were studied in this regard and cessation of menstruation occurred in four. Less distinct changes occurred in two. Menstrual cycles were unaffected in the remaining two. Hormone levels were assayed in a few patients but the methods were different and the results too sparse to report.

[6]This study will be reported in full detail in a separate paper by J. C. Heuson et al.

TABLE 1.—Combined tamoxifen-alternating cyclic chemotherapy: distribution of responses in fully evaluable patients

Dominant site of lesions	No. of—					Response (%)	
	CR	PR	No change	Progression	Total	CR	CR + PR
Soft tissue	9	3	1	4	17	53	71
Bone	1	4	1	2	8	12	62
Viscera	3	20	3	4	30	10	77
Total	13	27	5	10	55	24	73

Discussion and General Conclusions

The trial, based on concepts derived from ER studies and associating endocrine and cytotoxic therapies, gave encouraging results. A complete remission rate of 24% and an overall (CR + PR) remission rate of 73% had rarely been reported in studies involving strict criteria for assessment of response. Cycle A (ADM-VCR) alone was reported to achieve CR in 8% of the patients and CR + PR in 48%, the corresponding figures being 10% and 48% for cycle B (CMF) (11). Indeed, our results are not comparable to the latter since the two studies were performed in different, nonrandomized series of patients. Nevertheless it is suggested that the therapeutic regimen described here might be one of the most effective among those currently available. It will be of interest to have information on duration of remissions and survival, especially in view of the newly introduced alternating chemotherapy schedule.

If one tentatively admits that the apparent therapeutic superiority of the present regimen over ADM-VCR or CMF alone actually exists, it remains to be determined whether the difference is due to the concomitant administration of tamoxifen and chemotherapy, or to the use of two alternating cycles, or to both. The EORTC decided to compare CMF with CMF plus tamoxifen as the next step. CMF was chosen because it is simpler to administer and possibly less toxic than alternating chemotherapy and because it was found to be very effective in the adjuvant treatment of primary breast cancer (12). Assessment of the value of alternating cyclic chemotherapy should be the subject of a separate trial.

While tamoxifen was shown here to display therapeutic activity in premenopausal patients with advanced breast cancer, the magnitude of this activity was not assessed. The next step will be to compare tamoxifen with ovariectomy.

TABLE 2.—Effect of tamoxifen on menstruation

Patient No.	Age (yrs)	Mos. of tamoxifen administration	Menstrual cycles
1	40	1.5	Present
2	42	2.5	All missing
3	43	3	Present
4	45	3	1 delayed; 2 missing
5	45	13	All missing
6	46	4.5	Possibly delayed and decreased in volume*
7	52	3	All missing†
8	53	4	1 delayed; others missing†

*Massive uterine metastatic involvement.
†Already delayed before start of therapy.

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Proceedings of the US-USSR Joint Meeting on Lung Cancer

(USSR papers only)

Moscow, USSR

March 1974

This meeting was held as part of the US-USSR Chemotherapy Agreement under the US-USSR Agreement on Cancer Research. In an effort to expand the current knowledge on lung cancer research it was agreed that the USSR papers would be published in the US and the US papers would be published in the Soviet Union.

Cytologic Diagnosis of Lung Cancer

A. S. Petrova[1,*]

Contemporary cytologic diagnosis of lung cancer allows not only determination of the presence of tumor cells, but also differential diagnosis of the primary forms of lung cancer according to the most commonly used histologic classification.

In diagnosing lung cancer, a combination of cytologic methods of investigation are used, including (*a*) study of material from the primary focus (spontaneous or induced sputum; material produced upon bronchoscopy, including catheterization of the peripheral bronchii; transthoracic punctates), and (*b*) study of material allowing a judgment on the extent of disease spread beyond the lungs (pleural fluid; regional and peripheral lymph node punctates; punctates from metastatic foci in other organs).

Based on the characteristic signs determined in cytologic preparations, reliable cytologic diagnosis is possible for highly differentiated squamous-cell cancer (with keratinization), highly differentiated adenocarcinoma, and undifferentiated small-cell cancer.

Diagnosis of squamous-cell cancer with keratinization is based on the discovery of (*a*) cellular, functional, and structural signs of epidermoid differentiation, namely tumor cells reminiscent of epithelial cells; (*b*) their complexes, sometimes with intercellular bridges preserved; (*c*) characteristics of keratinization of the cytoplasm; and (*d*) cancerous "beads." This diagnosis should be made even if keratinization is observed in only a few tumor cells.

Indirect signs of squamous-cell cancer with keratinization include: (*a*) polymorphism of cells (small to gigantic in size, circular to irregular in shape); (*b*) polymorphism of hyperchromic compact nuclei; (*c*) significant numbers of cells in which the nuclei occupy a comparatively small portion of the cytoplasm; (*d*) absence of distinguisable nucleoli in most cells; and (*e*) "dirty" background on smear (unstructured amorphous mass, remains of nuclei and cytoplasm of keratinized and necrotic cells).

The diagnosis of highly differentiated adenocarcinoma is based on the discovery of cellular, functional, and structural signs of glandular differentiation, ie, polarity of cancer cells, manifested in eccentric placement of nuclei, and mucous formation and adenoid structures (rosettes, bubbles, papillae). If the mucus does not reveal recognizable signs by means of special dyes, an indirect but reliable sign of functional differentiation is detection of several or one large vacuole in the cytoplasm; the cells in this case resemble an inflated sphere or ring.

The criteria for cytologic diagnosis of undifferentiated small-cell cancer include: (*a*) absence in the tumor of cellular, functional, and structural signs of epidermoid and adenoid differentiation; (*b*) presence of small circular cells, slightly variable in size; (*c*) relatively large, centrally located hyperchromic nuclei with little variation in size; (*d*) presence of individual gigantic and usually deformed cells with hyperchromic nuclei, frequently in the form of "ink blots"; and (*e*) presence of compact accumulations in which the cytoplasm is not visible, "hollow" nuclei on neighboring surfaces having "facets."

In many cases a preliminary cytologic diagnosis is possible on the basis of signs discovered in the cytologic preparations for squamous-cell cancer without keratinization, alveolar carcinoma, and undifferentiated large-cell cancer. Diagnosis of squamous-cell cancer without keratinization can be made if cellular and structural signs of epidermoid differentiation are detected while the signs of keratinization are absent.

Among the predominant number of large cancer cells reminiscent of the cells of the prickle-cell layer (polygonal or circular cells with large nuclei and narrow rim of cytoplasm) a varying number of extended cells with fusiform nuclei are usually detected. The cells with different shapes give the picture a polymorphous appearance; sometimes complexes of polygonal or extended cells are seen with easily distinguished intercellular bridges. An important indirect sign is the presence of large and frequently multiple nucleoli in a large number of cells.

The criteria for the preferential cytologic diagnosis of alveolar carcinoma are as follows: monomorphic cylindric and cuboid cancer cells; manifest mucous formation; multiple fine papillar complexes of identical size; peripheral localization; no metastatic damage to the lungs; and absence of connection with different bronchii during bronchography. Clinical data can also be used in the diagnosis.

The criteria for a preliminary cytologic diagnosis

[1]USSR Academy of Medical Sciences, Moscow.

*Reprint requests to: Dr. A. S. Petrova, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

of undifferentiated large-cell cancer include (*a*) absence in the tumor of cellular, functional, and structural characteristics of epidermoid and adenoid differentiation; (*b*) predominance of large cancer cells, with little variation in size, significant (in comparison to cancers of other histologic structures) numbers of gigantic cells resulting in a polymorphous appearance of the picture, and occasionally a predominance of gigantic cells; (*c*) large hyperchromic or "bubble" nuclei, with little variation in size, located centrally and surrounded with moderately abundant cytoplasm; (*d*) absence of cancer cells and extended and fusiform nuclei; (*e*) formation of "facets" not contacting the cell surfaces; and (*f*) frequently "dirty" background on smear (abundance of amorphous detritus and decomposing nuclei and cells, including cell shadows with eroded contours).

Squamous-cell cancer with little differentiation and poorly differentiated adenocarcinoma have no reliable cytologic signs.

Histologic Classification of Lung Cancer

N. A. Kraevskii[1,*]

Lung cancer is one of the most common diseases of the lungs, as well as one of the most frequently encountered types of cancers. The successes of modern surgery have significantly expanded the capabilities of the morphologic study of lung cancer. This study is also facilitated by the more common use of bronchoscopic examination with biopsy. Cytologic study of bronchial smears and impressions produced in bronchobiopsy is now widely used.

Further progress in practical oncology requires both early and precise diagnosis, since it is otherwise impossible to select the most expedient method of treatment. The concept of precise diagnosis includes establishment of the nature, structure, histologic type, and degree of cataplasia of tumors. This sort of detailed characterization of the process has become particularly necessary in recent years because of the broad application of various antitumor preparations and new methods of radiation therapy. It is well known that the sensitivity to different treatment methods of tumors of various origins and degrees of maturity of the cell components differs greatly. The requirements for verification of tumors have increased greatly, with the primary significance given to morphologic study.

Having available extensive material on lung cancer from both operations and autopsies for a number of years (since 1966), we have utilized a classification of lung cancer based on the degree of differentiation and histogenetic type.

I. Squamous-cell cancer
   a. With keratinization
   b. Without keratinization
   c. Poorly differentiated

II. Adenocarcinoma
   a. Highly differentiated (acinous and papillary)
   b. Glandular-solid
   c. Poorly differentiated

III. Undifferentiated cancer
   a. Small-cell ("oat-cell")
   b. Small-cell ("lymphocyte-like")
   c. Large-cell

The group of undifferentiated cancers includes tumors which have no characteristics of functional and structural differentiation. Combined forms are encountered quite rarely.

This classification does not contradict the classification suggested by the World Health Organization but is simpler and more convenient to use, while the slight increase in the number of forms resulting from the biologic peculiarities of the tumors is not counterindicated.

After becoming familiar with the classification of lung cancer suggested by the VALCCSG, we concluded that it basically coincides with that which we use. One variation is found in the classification of small-cell undifferentiated cancer. We do not classify into a separate group the tumors with the polygonal cell structures, since these tumors in our classification are looked upon as poorly differentiated adenocarcinoma on the basis of their formal morphologic characteristics (judging from the slides presented). The group of poorly differentiated cancers is the most difficult for diagnosis, as well as the large-cell undifferentiated cancers. The latter are, obviously, adenocarcinomas.

After analyzing the available materials, we can conclude that there are no basic differences in the classifications which we use and those used by others, and we need only find a common language for the evaluation of controversial forms, without allowing subjective decisions in their perception and interpretation. This exchange of opinions will be mutually helpful and doubtless will facilitate further fruitful work.

[1]USSR Academy of Medical Sciences, Moscow.
Reprint requests to: Dr. N. A. Kraevskii, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

Occurrence of Lung Cancer According to Stage and Determination of its Dissemination

A. I. Pirogov,* S. Ia. Marmorshtein, I. A. Maksimov, and V. V. Starinskii[1]

Despite a certain pattern in the characteristics of the development of lung cancer, the primary tumor, and the metastatic process, there are a number of inadequacies in the four-stage classification system and in the use of the TNM system. They relate to the lack of a clear-cut division of the stages of the disease, the absence of a concept of the character of the neoplastic growth, the absence of sufficiently complete characterization of the metastatic process and the involvement of individual groups of lymph nodes and organs in the neoplastic process, and the lack of information about the histologic structure and degree of morphologic differentiation of the neoplastic tissue.

It is difficult to overestimate the importance of all this information if we accept the present-day need for more in-depth study of the intra- and extra-organ dissemination of the neoplasm in order to plan not only surgical and radiation therapy, requiring treatment both of the basic neoplastic nodules and the zones of regional metastasis, but also rational chemotherapy. These data are also essential for an assessment of the effectiveness of the various therapeutic measures and the final results of the treatment, ie, the prognosis.

A more extensive characterization of the neoplastic process is possible only through a more exact determination of the stages with detailed values for the TNM symbols.

Current methods for determining the extent of lung cancer and its diagnosis are based on initial information about the tumor and its histologic structure. From among these methods, we should select those that enable us to obtain sufficiently complete information on the size of the neoplasm, the probable zones of subclinical spread, and the degree of implication of the lymphatic collectors of the zones of regional metastasis and remote organs, with minimal waste of time and loss of the patient's strength.

Although the size of the tumor and the volume of the damage within the limits of the segment or lobe can be adequately determined in peripheral cancer by roentgenographic and tomographic data, this process is considerably more complicated in the central form of the cancer, since it is practically impossible to establish the dimensions of the tumor itself because of the accompanying atelectasis or pneumonitis and the enlargement of the bronchopulmonary lymph nodes. The difference between a tumor limited to a lobe or one passing into a major bronchus can be clearly determined by tomographic data, whereas when there is involvement of a segmental bronchus the apparent extent of dissemination is not always evaluable and may be exaggerated. Thus, endoscopic confirmation of the extent of spread is essential in cases of central cancer, providing an evaluation that is complete in 92% of those with this form of tumor and reflecting the real extent of the local damage. The bronchoradiometric method using a radioactive indicator labeled with the isotope ^{32}P makes it possible to characterize the level of phosphorus metabolism in the focus of the lesion and is very important not in determining the intra-organ distribution of the tumor but in differential diagnosis.

Tomographic studies reveal enlargement of the lymph nodes in > 90% of the patients. This reliable judgment also applies to individual groups both on the side of the tumor and on the contralateral side. Functional upper cavagraphy or bilateral mediastinal phlebography has definite value in detecting enlarged right paratracheal lymph nodes not determined in roentgenography or even tomography. In 35% of the cases, however, the enlarged lymph nodes were found to be only hyperplastic, without metastases. This high percentage of hyperdiagnosis results in an unjustified rejection of radical surgery and an unobjectively optimistic evaluation of tumor regression with conservative therapy. Thus, the value of roentgenologic signs of enlargement of lymph nodes consists in their reliability and the determination of the groups for which additional study with morphologic confirmation of the metastatic character of their disease is indicated.

The significance of the endoscopic method in determining metastases in individual groups of lymph nodes is only modest, and the conclusion is based largely on the indirect signs of deformation and the

[1]Department of Thoracic Oncology, The Herzen Oncological Institute, Moscow, USSR.

*Reprint requests to: Prof. A. I. Pirogov, Department of Thoracic Oncology, The Herzen Oncological Institute, Moscow, USSR.

limitation of respiratory mobility of the walls of the bronchi and trachea. Transbronchial puncture biopsy, evaluated in the later stages (III and IV) of the disease, makes it possible to discover cancer cells in the subsequent histologic investigation in 30% of the patients.

The effectiveness of transbronchial puncture biopsies depends on the localization of the tumor with its inherent patterns of metastasis and histologic structure. Thus, metastatic involvement of the lymph nodes is 1.5 times as common in cases of undifferentiated cancer as in the differentiated forms.

Surgical diagnostic methods occupy a special place in the determination of the distribution of a tumor within the thoracic cavity. Although prescalene biopsy has retained its importance among these methods only in cases of enlarged, palpable supraclavicular lymph nodes, the role of mediastinoscopy and parasternal mediastinotomy is considerably broader. At the same time, despite their high degree of effectiveness, these represent methods of final diagnosis, used when other simpler methods cannot give sufficient information about the nature of the enlargement of the mediastinal lymph nodes. Their use is indicated in cases of morphologically confirmed lung cancer when there is doubt of the possibility of radical surgery or histologically determined poorly differentiated lung cancer, and is also indicated when there is a suspicion of this form of cancer as a result of rapid progression of the disease with enlargement of the mediastinal lymph nodes. Use of these methods makes it possible to detect metastases in the mediastinum in 50.8% of the patients in whom there was reason to suspect them, and in 75.6% of the patients with poorly differentiated cancer, reducing the number of exploratory thoracotomies to 5%.

The combination of roentgenologic study and parasternal mediastinotomy is the most reliable method for judging the condition of the intrathoracic lymph nodes in 97% of lung cancer patients.

In connection with the detection of remote metastases of lung cancer (cerebrum, adrenals, liver, kidneys), our capabilities are limited by the complexity of the methods of study, most commonly angiographic, which are not easily tolerated by weakened patients in the last stages of their disease, and by the inadequacy of the information obtained with the other more usual study methods. Approximate data on the functional state of the liver is given by radioisotope hepatography with ^{131}I-Bengal rose, and radioisotope scanning with ^{198}Au yields information about its focal damage. Laparoscopy with puncture biopsy of diseased areas and even laparatomy may be indicated for the detection of metastases in the liver in some patients for planning conservative methods of treatment. It is not possible, of course, to judge the real character of the pleural exudate without cytologic examination.

Thus, none of the methods of study has a decisive role in determining the extent of spread of lung cancer, and only a combination of methods, used according to clinical indications, will enable us to judge with considerable certainty the actual extent of the neoplastic process. Further refinement of the symbols of the TNM system as a result of the information obtained will make it possible to characterize the stage of cancer, select the most appropriate therapeutic method, and evaluate objectively its effectiveness, prognosis, and immediate and long-range results.

General Principles of Surgical Treatment of Lung Cancer

S. Ye. Peterson[1,*]

This report presents the general principles of the surgical treatment of lung cancer, avoiding problems still in the stage of scientific development. It outlines the view of surgical treatment of this disease accepted in the USSR and some observations from the surgical clinic of the Institute of Experimental and Clinical Oncology, representing a part of this collective experience.

Russian surgeons have kept abreast with the surgeons of other countries in the search for surgical methods of treatment of lung cancer. In 1905, F. R. Kievskiy published a book entitled "The Study of the Resection of the Lungs." This work was significant in the development of pulmonary surgery, since it briefly outlined the need for separate treatment of the root of the lung in operations on the lungs, and these positions formed the basis for modern techniques of pneumonectomy. In 1902, V. M. Chekan described pulmonary resection with growth of a pectoral wall sarcoma into the lungs. This was the 12th case of pulmonary resection described in the world literature.

From 1902 to 1935, Russian surgeons met regularly to discuss problems of pulmonary surgery. Following the lead of E. A. Graham, who in 1933 performed the first pneumonectomy in a patient with cancer under local anesthesia, A. V. Vishnevskiy successfully repeated this operation in 1937 using local anesthesia. In 1940, L. K. Bogush successfully performed two lobectomies in succession. Since that time, lung cancer surgery has continually progressed and has become a routine method in the treatment of this disease in our country.

Physicians are obligated to ask permission to perform surgical treatment in patients with pulmonary carcinoma and to exhaust all existing diagnostic methods in order to determine the true status of the disease; surgery should not be refused without reason. This is because, at the present time, only surgery can offer the patient hope of total recovery.

We always look for two types of contraindications to surgery for lung cancer. These are oncologic contraindications and contraindications based on a combination of factors which may increase the severity of surgery: advanced age, accompanying diseases, and low functional indicators. We mention these factors in combination since individually each of these is frequently not sufficient as a basis for refusal to perform surgery. At the present time, a tendency has clearly developed toward narrowing the indications for surgery based on oncologic indicators and expanding the indications for surgery in persons with factors which may increase the severity of the surgery.

Unfortunately, the opportunities to perform radical surgery are depressingly few, and apparently about the same in various countries. For example, according to our data, in Moscow the percentage of patients first admitted and later found to be operable averages 10%–12% in different years.

The decision to use either pneumonectomy or lobectomy depends on the stage of the disease process and the condition of the damaged bronchus. Lobectomy is most frequentiy performed in patients with peripheral cancer of the lungs and pneumonectomy is most frequently performed in patients with central cancer. Each type of operation has its own specific indications, which are determined on the operating table. In recent years, the percentage of lobectomies has increased not only because of the improved diagnosis of lung cancer, but also because of the refinement of indications for this operation.

In the performance of pneumonectomies and lobectomies we concentrate our primary attention on removal of lymph nodes. In typical pneumonectomies, the group of bifurcational, tracheobronchial, and lower paratracheal lymph nodes is removed. In superior lobectomies, we sometimes do not remove the group of bifurcational nodes, particularly if the upper portion of the right lung is removed. When the lower portions of the lung are removed, the bifurcational nodes are always removed. Combined resection in patients with broad local spread of cancer to neighboring anatomic formations (chest wall, diaphragm, etc) does not assure survival for > 5 years and therefore generally should not be performed. There are various points of view concerning the sequence of treatment of elements of the root of the lung. However, most clinics, including ours, consider that the intensity of metastasis depends not so much on the spread of cancer cells through

[1]Cancer Research Center, USSR Academy of Medical Sciences, Moscow.

Reprint requests to: Prof. S. Ye. Peterson, Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

the blood stream as on special unfavorable immunobiologic situations in the organism. Based on this, we use the multistage technique and treat the elements of the root of the lung in a sequence assuring minimum trauma to the tumor. Our domestic suturing apparatus (UKL, UKD, etc) allows rapid, low trauma, and reliable surgery. We use these devices widely in separate treatment of elements of the root of the lung.

We recognize broad indications for surgery in patients with factors which might increase operational severity, and our postoperative death rate is approximately 8% (lower for lobectomies). The postoperative death rate is not significantly decreased by expanding the indications for surgery in patients with factors increasing severity. The causes of death in our patients are apparently the same as those experienced by surgeons in other countries. We attach great significance to assuring a favorable outcome in older patients and patients with accompanying diseases increasing the chance for severity, providing the maximum active postoperative period for these patients. Epidural anesthesia is widely used in the postoperative period.

The results of surgical treatment in various groups of patients with carcinoma of the lung are different. Dependence on the stage of the disease process is generally known. The results differ for patients in whom lung cancer has been diagnosed during routine chest roentgenogram and for patients who have independently come in for treatment during the period of development of clinical cancer. They differ also for different histologic forms of the disease.

There is great interest in determining the true radicality of surgery. The surgical clinic of our institute has studied the results of autopsies of patients who have died after so-called radical operations for lung cancer due to postoperative complications. In one third of these autopsies, the pathologist has found metastases not noted by the surgeon during the operation.

Analysis of the current results of surgical treatment of lung cancer leads to the following three main conclusions: (*a*) surgical methods can be used in a small number of lung cancer patients (10%); (*b*) truly radical operations are performed for only about one third of surgical patients who survive >5 years; and (*c*) with squamous-cell cancer and adenosarcoma the indications for surgery can be expanded, and with undifferentiated lung cancer the indications for surgery should be restricted.

All of this indicates that our main thrust in the search for methods of treatment should be in the direction of chemotherapy.

Prerequisites for Effective Radiotherapy for Lung Cancer

A. I. Ruderman[1,*]

Radiotherapy is one of the basic and quite radical treatments for malignant tumors in many sites of the early disease and is effective palliative treatment for late stages of the disease. It was not so long ago that radiotherapy was being used in approximately 50% of cancer patients. Recent USSR data show that radiotherapy is given to > 70% of patients with malignant neoplasms.

Approximately 15%–20% of the cancer patients given radiotherapy have lung cancer and in these patients it is customary to use different irradiation variants. Patients selected to receive radical radiotherapy are preferably patients on whom radical surgery could be performed but who refuse to undergo surgery, or patients whose health contraindicates radical surgery. Radiotherapy must be regarded as a palliative measure in all other patients.

A summary of the results of the use of radiotherapy alone in connection with lung cancer, published by Soviet authors from 1956 to 1971 and including 1270 patients, indicates that the 5-year survival rate averaged 7.5% (table 1). This response rate must be regarded as unsatisfactory once one is aware that this group includes those with histologically unconfirmed diagnoses. These factors are precisely why there has been a loss of interest in radiotherapy as an independent method for treating lung cancer and why there have been active searches for different chemical agents to combine with radiotherapy.

This paper will outline some of the organizational and methodologic conditions under which radiotherapy can have a favorable influence on the effectiveness of combination chemotherapy/radiotherapy, disregarding the question of the specific chemical agents with which irradiation should be combined. Collective experience, gained in the course of independent studies of radiotherapy for malignant lung tumors, as well as data from a detailed study of the histories of patients with long periods of survival after treatment, are the basis for the considerations to be outlined below. The concept that regardless of its original histologic characteristics lung cancer is primarily a localized tumor with, most frequently, early lymphogenous metastatic spreading, which, under favorable conditions, can be cured by a single exposure to radiation, proved to be important in the formulation of the following considerations:

[1]Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, Moscow.

*_Reprint requests to:_ Dr. A. I. Ruderman, Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

TABLE 1.—Combined results of radiotherapy for lung cancer

Investigator	Year	Type of radiation	No. of patients	% survival for years—				
				1	2	3	4	5
A. V. Kozlova	1956	^{60}Co	56	29	11	—	5	—
M. M. Nivinskaya	1957	X-ray (200 kev)	239	27	25	8	2	2
T. N. Matveyeva	1964	^{60}Co	116	47	20	12	7	3
M. M. Beybutov and V. S. Golynskaya	1965	^{60}Co	170	35	—	—	—	—
I. A. Pereslegin	1966	^{60}Co	364	12	8.2	6.8	—	—
A. S. Yachkevichus	1969	^{60}Co	142	38.8	17.1	10.0	6.8	6
A. S. Strashinin	1972	X-ray (200 kev) or ^{60}Co	70	58.6	24.3	15.7	15.3	14.5
A. S. Strashinin	1971	High-energy inhibiting radiation, 4 Mev	113	61	35	21.9	23	14.8
Totals			1270	38.5	20.1	12.4	9.8	7.5

1. Strict selection of patients for radical radiotherapy must be carried out (this is possible only with respect to patients still with the localized stage of the disease);

2. There must be a reasonably accurate determination of the volume of the tissue to receive irradiation (the "target"), including the apparent focus of the lesion (radical irradiation), and a determination of the regional zones of possible metastatic spread (prophylactic irradiation);

3. The volume of tissue subjected to radical radiation treatment should include the visible primary tumor and determinable lymphogenous metastases of stages I (bronchopulmonary nodes of the hili of the lung), II (tracheobronchial), and III (paratracheal and mediastinal), whereas prophylactic irradiation should involve those volumes containing all of the apparently unaffected intrathoracic tissue and the regions of the supraclavicular and scalene lymph nodes;

4. Random radical radiotherapy is administered to lung cancer patients upon the development of lymphogenous metastases beyond the limits of the stages listed in paragraph 3, as well as when hematogenous, mixed (lymphogenous and hematogenous), or aerogenous (aspiratory-bronchogenic) metastases are present (only palliative irradiation, in combination with chemotherapy, is recommended for these patients);

5. The most desirable types of conventional radiation to use are high-energy inhibiting radiation or cobalt 60 gamma radiation. This is based on the proposition that the damaging effects of the different types of radiation are identical when the relative biologic effectiveness is taken into consideration, something that, all other conditions being equal, depends uniquely on the magnitude of the absorbed dose as well as on the fact that the degree of radiation trauma to the healthy tissue surrounding the tumor is definitely associated with the physical characteristics of the incident radiation and the procedure used to get the radiation to the target. This will, at the same time, cause fewer radiation complications and less evidence of such complications involving the surrounding healthy tissue;

6. Static, as well as mobile, variants of irradiation can be used. At the same time, the single and total doses, and the break between treatments, should be corrected for target volume using the calculation of the magnitudes of the nominal standard dose adopted for this purpose or the rad equivalent. The following values for the latter are desirable for radical radiotherapy for malignant lung tumors of various histologic structure:

Histologic type	Nominal standard dose	Rad equivalent
Epidermal	1650–1750	5500–6000
Glandular	1750–1880	6000–6500
Undifferentiated	1450–1650	4500–5500

The radiation load for prophylactic irradiation should be 75% of the dose selected for radical irradiation;

7. In the case of a central cancer, it is first desirable to irradiate one field with the dose as palliative radiotherapy, and then as the course of radiotherapy proceeds, to reach a dose of 100%. Irradiation in this manner can rarely be used in the case of a peripheral cancer;

8. Recognizing the present-day successes of chemotherapy, it is obviously desirable to use special drugs to supplement independent radical treatment of a patient with lung cancer, in certain cases, after the histologic structure of the tumor has been taken into consideration. Combination chemotherapy/radiotherapy should be recommended in all cases of palliative treatment. It can be assumed that the end result of combination treatment will be the destruction of all malignant structures in the region of the local application of radiation as a result of the combined effect of both therapeutic agents (radiation plus chemotherapy). In addition there should be a disruption in the life cycle of the cells and, in particular, a disruption in the capacity of pathologic formations scattered outside the region of irradiation to proliferate, attributable to the chemotherapy;

9. The requirements for drugs used in combination treatment are based on the foregoing. These drugs should, regardless of the mechanism involved in their effect, independently cause persistent injury to disseminated cancer complexes, using doses that can be tolerated by the patient. It may be assumed in advance that these doses will cause less damage to the main tumor, the volume of which is much larger than the metastases;

10. It is extremely important that radiotherapy and combination treatment not be regarded as the only possibility for treatment of lung cancer. Steps should be taken to restore the immunoreaction systems in the body of every patient suffering from cancer, radiation trauma, and drug effects, and a

variety of prophylactic measures should be used to overcome the complications caused by accompanying infection and reactions to radiation by different organs and tissues. The use of corticosteroids, vitamins, antibiotics, and other means is suggested.

These considerations are, from our point of view, the prerequisites for effective radiotherapy. They should be kept in mind when devising the plan for treating a specific patient with a malignant lung tumor.

Chemotherapy for Lung Cancer

N. I. Perevodchikova[1,*]

Chemotherapy for lung cancer still remains one of the most urgent and, at the same time, difficult problems in the medical treatment of cancer. Because of unsatisfactory results from surgical and radiation treatment of lung cancer and especially because the diagnosis is often established at late stages, the question of whether to use chemotherapy is usually considered at some time during the treatment of most patients with this form of malignant tumor. However, the available types of modern chemotherapy for lung cancer are extremely limited.

Using as an example the 30 years of experience of the Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, this paper will present the results of chemotherapy in 465 patients with inoperable lung cancer (table 1). A positive objective effect was considered a roentgenologic reduction or disappearance of the shadow on the lung, a bronchoscopically determined reduction of the tumor, and a reduction or disappearance of remote metastases. Complete disappearance of the tumor or its reduction by > 50% was given a rating of 3, reduction of the tumor by 25%–50% was rated as 2, and reduction by < 25% was rated as 1.

As seen in table 1, the most effective drugs studied were such well-known preparations as mechlorethamine and cyclophosphamide, and of the new drugs the greatest effect was given by 1-methyl-1-nitrosourea (MNU)—a drug prepared as an antitumor agent by our institute.[2] During the last 2 years we have concentrated on the effect of this latter drug in lung cancer. After establishing that small-cell lung cancer was quite sensitive to the drug and that there was a lack of cross resistance between MNU and cyclophosphamide, we began to study the use of MNU combined with cyclophosphamide. We found that these two drugs given in repeated 3-week courses (iv infusion of 300 mg of MNU twice a week and 400 mg of cyclophosphamide three times a week) produces a pronounced objective effect; ratings of 2 and 3 were obtained in 60% of the patients with small-cell lung cancer and 26% of the patients with squamous cell lung cancer. The average length of remission was 2.6 and 1.8 months respectively (table 2).

Side effects and complications from this combination of MNU and cyclophosphamide are given in table 3.

The combination does not increase the frequency

[1]Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, Moscow.

*_Reprint requests to:_ Prof. N. I. Perevodchikova, Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

[2]Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5.

TABLE 1.—Results of chemotherapy for lung cancer

Drug	No. of patients treated	No. of patients with objective effect— 3	2	1	Total	%
Mechlorethamine	98	2	10	12	24	24
Cyclophosphamide	80	1	13	4	18	22
ThioTEPA and other ethyleneamines	58	—	1	2	3	5
MNU*	37	13	8	3	24	65
Cyclophosphamide + MNU	49	7	12	4	23	47
Sarcolysin and its peptides	42	—	—	1	1	2
Antitumor antibiotics†	33	—	—	3	3	9
Antimetabolites‡	17	—	—	—	—	0
Polychemotherapy	61	2	7	3	12	20
Totals	465				115	24.6

*Used primarily with small-cell cancer.

†Ribomycin, olivomycin, chrysomalin, and mitomycin C.

‡5-Fluorouracil and methotrexate.

TABLE 2.—Results of the use of a combination of MNU and cyclophosphamide

Type of lung cancer	No. of patients treated	No. of patients with objective effect— 3	2	1	3 + 2 (%)	Length of remission (mos)
Squamous cell	19	1	4	2	26	1.8
Small-cell	20	5	7	2	60	2.6
Large-cell	3	—	—	—	0	
Adenocarcinoma	1	—	—	—	0	
Undetermined form	6	1	1	—	33	1
Totals	49	7	12	4	40	

TABLE 3.—Side effects and complications from MNU and cyclophosphamide in 49 patients

Effect	% of total patients
Leukopenia (3000–2000 cells/mm³)	16
Leukopenia (2000–1000 cells/mm³)	14
Thrombocytopenia (120,000 cells/mm³)	6
Nausea, vomiting	80
Phlebitis	8
Diarrhea	2
Cystitis	2
Fever	2

of remission in small-cell cancer of the lung compared to MNU alone (60% and 72% remissions respectively). However, results obtained with the use of this combination in patients with squamous cell lung cancer (26% response rate) indicate definite prospects for further study of its use. On the basis of these data, as well as studies by American investigators to determine the effectiveness of CCNU in cancer of the lung (17%–33% objective responses), cooperative studies were organized through the All-Union Chemotherapeutic Antitumor Center which compared the effectiveness of our schedule with a combination of CCNU and cyclophosphamide. These studies, in which nine institutions in our country are taking part, began recently and therefore the results cannot be evaluated as yet.

In judging the results of chemotherapy for lung cancer, we must first note the clear dependence of the effectiveness of treatment on the histologic type of lung cancer (table 4). Most sensitive to chemotherapeutic effects is small-cell lung cancer; the best results, according to our data, are given by the use of MNU alone (72% effectiveness) and MNU in combination with cyclophosphamide (70% effectiveness), while mechlorethamine alone was effective in 48% and cyclophosphamide alone was effective in 17% of the patients with this type of cancer.

Squamous cell cancer is much less sensitive to chemotherapeutic drugs (responses occurred in 14 of 124 patients, ie, 11%). However, there was a relatively higher effectiveness of the combination of MNU and cyclophosphamide in this type of lung cancer (responses in seven of 19 patients, ie, 36%; in five patients [26%] the effect was rated as 2 or 3). Finally, it must be noted that we observed practically no positive effect from chemotherapy in cases of adenocarcinoma of the lung.

The overall survival of patients with various histológic types of lung cancer treated with chemotherapy is given in table 5. The average survival of patients with various histologic types of lung cancer according to the treatment response is given in

TABLE 4.—Results of chemotherapy according to various histologic types of lung cancer

Drug	Squamous cell (124 patients)	Adenocarcinoma (39 patients)	Small-cell (118 patients)	Large-cell (10 patients)	Undetermined form (74 patients)
	2/31 (6%)	0/6	10/21 (48%)		5/8 (62%)
Cyclophosphamide	2/23 (9%)	0/12	4/23 (17%)	0/1	3/11 (27%)
Ethyleneamines	0/6	0/6	0/5	0/2	1/7 (14%)
Sarcolysin and its peptides	0/8	0/3	0/6	0/1	1/11 (9%)
MNU	—	0/1	21/29 (72%)	1/2 (50%)	2/5 (40%)
MNU + cyclophosphamide	7/19 (36%)	0/1	14/20 (70%)	0/3	2/6 (33%)
Antitumor antibiotics	0/8	0/3	0/4	—	1/8 (12%)
Antimetabolites	0/10	0/3	0/1	—	0/2
Combination chemotherapy	3/19 (15%)	1/4 (25%)	2/9 (22%)	1/1 (100%)	2/16 (12%)
Totals	14/124 (11%)	1/39 (2.6%)	51/118 (43%)	2/10 (20%)	17/74 (23%)

TABLE 5.—Survival of patients receiving chemotherapy according to histologic type of lung cancer

Type of lung cancer	% of patients surviving—			
	3 mos	6 mos	12 mos	≥24 mos
Squamous cell	72	43	17	5
Adenocarcinoma	48	34	5	—
Small-cell	62	30	6	1
Large-cell	80	25	—	—
Undetermined form	75	40	16	2

TABLE 6.—Survival of patients according to effect of treatment and histologic type of lung cancer

Type of lung cancer	No. of patients treated	No. with effective treatment	Average survival (mos)	
			Effective treatment	No effect
Squamous cell	124	14 (11%)	12	6
Small-cell	118	52 (43%)	6.7	2.9
Adenocarcinoma	39	1 (2.6%)		
Large-cell	10	2 (20%)	7.5	4
Undetermined form	74	17 (23%)	7.4	6
Totals	365	85 (23.5%)	8.7	5

table 6. The average survival of the patients is determined to the greatest degree by the histologic variant of lung cancer; however, the results of chemotherapy are undoubtedly reflected in patient survival. Patients with squamous cell and small-cell cancer of the lung who reacted positively to chemotherapy lived twice as long as patients in whom chemotherapy was ineffective (12 and six patients, 6.7 and 2.9 months average survival, respectively). This indicates a more optimistic attitude toward chemotherapy for lung cancer; expansion of such treatment depends on the discovery of new, more active antitumor drugs, various rational combinations of these agents, and the use of chemotherapy as one component in multi-modality therapy for lung cancer.

Conservative Treatment of Patients With Small-Cell Lung Cancer

I. V. Kas'yanenko[1,*]

We examined 373 patients with inoperable lung cancer in stages III and IV who had received combination chemotherapy/radiation or chemotherapy alone. Diagnosis was made on the basis of careful clinical-roentgenologic examination, bronchoscopy, bronchography, or exploratory thoracotomy. Diagnosis was verified by a morphologist in all patients. General characteristics of treated patients are given in table 1.

As can be seen from table 1, 179 patients received chemotherapy and 194 received chemotherapy/radiation. Small-cell cancer was established in 155 patients. The immediate effect of chemotherapy/radiation was directly related to the radiation dose, the chemotherapeutic drug, and the stage of the disease.

Objective effect was defined as disappearance of the tumor or a 25%–50% reduction of its size, disappearance of regional lymph nodes, and restoration of broncho-pulmonary passage. The effect was also evaluated on the basis of clinical, roentgenologic, spirographic, and in some patients bronchoscopic examinations.

The direct results of chemotherapy/radiation of patients with small-cell cancer are given in table 2. An objective effect was observed in 40 patients and a subjective effect in only 24. The best objective effect was found with the combination of tele-gamma-therapy ("Rocus" equipment) and cyclophosphamide (66% ± 10.1% of the patients).[2]

The results of treatment were also evaluated by two more indices: survival every 6 months after the end of treatment and average survival; these were slightly better with the combination of tele-gamma-therapy and cyclophosphamide. The average survival of patients with small-cell cancer was 10.1 months; in the control group of patients, who received only tele-gamma-therapy, survival was the shortest, 5.9 months. In patients with differentiated forms of cancer the highest average survival (up to 17 months) was observed with a combination of tele-gamma-therapy and 5-fluorouracil.[3]

Chemotherapy alone was given to 74 patients with small-cell cancer of the lung. This group was characterized by pronounced general intoxication, significant distribution of the disease process, tumor decay, and pleuritis. The patients were treated with a combination of two or three drugs with various mechanisms of action. Results of polychemotherapy in patients with small-cell lung cancer are shown in table 3. As this table shows, the frequency of an objective effect with small-cell cancer did not depend on the kind of chemotherapy and was observed in approximately one third of the patients.

Subjective improvement was achieved in 48 of 74 patients. With differentiated forms of cancer, an objective effect was achieved slightly more frequently with three cytostatic agents (an alkylating agent, an antimetabolite, and vinblastine).[4] Chemotherapy did not considerably prolong the life of patients, especially those with small-cell cancer where the average survival was 3.8–4.4 months.

The general toxicity from polychemotherapy was studied in patients with small-cell and in those with

[1]Institute of Problems of Oncology, Ukrainian SSR Academy of Sciences, USSR.

[2]Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

*_Reprint requests to:_ Dr. I. V. Kas'yanenko, Institute of Problems of Oncology, Ukrainian SSR Academy of Sciences, Vasilkovskaja, 45, 252127, Kiev-127, USSR.

[3]5-Fluorouracil: NSC-19893; CAS reg. No. 51–21–8.

[4]Vinblastine: NSC-49842; CAS reg. No. 6449–03–2; vincaleukoblastine, sulfate (1:1), monohydrate.

TABLE 1.—General characteristics of patients with lung cancer given conservative treatment

Treatment	No. of patients treated	Sex		Location of cancer		Stages		Morphologic structure of tumor		
		Male	Female	Central	Peripheral	III	IV	Small-cell carcinoma	Epidermal carcinoma	Adeno-carcinoma
Chemotherapy	179	155	24	145	34	106	73	74	81	24
Chemotherapy/radiation	194	176	18	159	35	142	52	81	87	26
Total	373	331	42	304	69	248	125	155	168	50

TABLE 2.—Direct results of chemotherapy/radiation of small-cell lung cancer

Treatment	Doses	Total No. of patients treated	Effect		
			Objective	Subjective	None
Tele-gamma-therapy + Cyclophosphamide	3000–8000 rads 3.0–8.0 g	21	14 (67.7%)	4	3
Tele-gamma-therapy + 5-Fluorouracil	3000–8000 rads 3.0–8.0 g	20	9 (45%)	5	6
Tele-gamma-therapy + Methotrexate*	3000–8000 rads 60–140 mg	22	10 (45%)	7	5
Tele-gamma-therapy	2000–8000 rads	18	7 (39%)	8	3
Total		81	40 (49.3%)	24	17

*Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

TABLE 3.—Direct results of chemotherapy of small-cell lung cancer

Treatment	Doses	No. of patients treated	Effect		
			Objective	Subjective	None
Cyclophosphamide + Methotrexate or (5-Fluorouracil)	4.0–6.0 g 40–60 mg (3.0–4.0 g)	25	7 (28.0%)	9	9
Cyclophosphamide + Methotrexate or (5-Fluorouracil) + Vinblastine	4.0–6.0 g 40–60 mg (3.0–4.0 g) 30–40 mg	24	8 (33.3%)	10	6
Cyclophosphamide	6.0–8.0 g	25	7 (28.0%)	7	11
		74	22 (29.7%)	26	26

differentiated cancer. Generally, moderate depression of myelopoiesis was detected after the end of treatment. The number of neutrophils and lymphocytes in the peripheral blood was reduced to 53% and 41% respectively.

Indices of cellular immunity were studied by the reaction of suppressing hypersensitivity to dinitrochlorobenzene.[5] Before treatment this reaction was positive in only 14% of the patients with lung cancer, while after treatment, especially after an objective effect, the reaction became positive in 28% of the patients.

Histochemical and morphologic studies of the mucous membrane of the stomach revealed signs of chronic gastritis in a significant number of patients with lung cancer. After treatment we noted a reduction in the intensity of RNA and DNA synthesis and mucopolysaccharides and increased signs of dystrophy in the mucosa of the stomach; this was also shown in experiments on dogs with fistulas of the fundal and pyloric sections of the stomach.

Studies with ^{131}I-labeled hippuran showed disturbance of the excretory function of the kidneys which did not appear clinically. Renogram curves were characterized by a smoothed peak of maximum concentration of the isotope and reduction of the excretory phase. Changes in renograms depended on the effectiveness of treatment. With a positive

[5]Catalona WG, Taylor PT, and Chretien PB. Clin Exp Immunol 12:325–333, 1972.

effect renograms showed improvement and a tendency toward normalization of the excretory function of the kidneys. Deterioration of renogram curves was noted with ineffective treatment.

Generally, deterioration of coronary circulation was noted on the electrocardiogram in patients with a history of cardiovascular disease.

In conclusion, it can be stated that moderate complications developing during treatment are usually cured by symptomatic agents. The doses and combinations of antineoplastic agents we used are completely suitable for treatment of these patients.

The results of conservative treatment of patients with lung cancer, including small-cell cancer, are, unfortunately, still unsatisfactory and require further searches to improve methods of treatment.

Experience With Combination Chemotherapy and Radiotherapy for Undifferentiated Lung Cancer

A. I. Ruderman,* V. S. Kiseleva, M. M. Nivinskaya, M. S. Starichkov, and S. L. Dar'yalova[1]

Undifferentiated lung cancer is characterized by rapid growth of the primary tumor, early dissemination, and a poor prognosis. In most patients the first clinical symptoms of the disease appear after the tumor has advanced beyond the limits of the primary focus, when the intrathoracic lymph nodes have already been damaged, and when there are sometimes distant metastases. Because of this biologic peculiarity local treatment methods are unsuccessful. It is precisely for this reason that a search was made for a combination of methods that would have an effect on the primary tumor, the regional lymph nodes, and the subclinical dissemination.

This report is devoted to a retrospective analysis of the results of our combination therapy for undifferentiated lung cancer. One hundred patients (94 men and six women), 23–74 years of age, were treated between 1960 and 1972. Of these patients, 42 (42%) were < 50 years of age. The extent of the disease was stage III–IV in all patients. A central tumor was recognized in 73 patients and a peripheral tumor in 17. The original localization of the tumor was not established in ten patients because of the extent of the disease process. Morphologic study confirmed the diagnosis of undifferentiated cancer in all cases.

This report does not break down the undifferentiated cancer into its morphologic subtypes, although the fact that the different morphologic variants do not respond similarly to identical treatment is not excluded.

Patients received combination chemotherapy and radiotherapy. Twenty-three patients received radiotherapy and 5-fluorouracil[2] and 77 received radiotherapy and cyclophosphamide.[3] Radiotherapy was administered using distant gamma-therapy equipment with a cobalt 60 source. Two fields (anterior straight ahead and posterior at an angle of 15°–30°) or three fields (anterior, posterior, and lateral) were used for irradiation. The primary tumor and the region of the mediastinum were within the irradiation volume. The supraclavicular regions were subjected to radiation only when there were clinically established or suspected metastases. Irradiation treatment followed the conventional fractionation scheme (five or six sessions per week). The weekly local dose was 1000–1200 rads. Total local doses were different, ranging from 3000 to 6000 rads.

When 5-fluorouracil was used, it was injected at a dose of 250 mg iv 30 minutes prior to each irradiation session. The total dose of the drug per course was 3.0–6.0 g. When cyclophosphamide was given, it was injected at a dose of 15 mg/kg im or iv once a week. The total dose per course was 3.0–7.0 g.

The following results were recorded immediately after the completion of a course of combination therapy: objective improvement 70%, subjective improvement 21%, and disease progression 9%. Definitions of response are as follows:

1. *Objective improvement.*—Disappearance of or significant reduction in the clinical symptoms of the disease; roentgenologically documented complete or clearly expressed regression of the tumor and metastases.

2. *Subjective improvement.*—Disappearance of or reduction in the clinical symptoms of the disease (fever, cough, pain, etc) in the absence of persuasive roentgenologically documented regression of the tumor and metastases.

3. *Disease progression.*—Evidence of progression or no effect.

Table 1 lists the results of the therapy. The greatest objective effect was achieved with the combined use of radiation and cyclophosphamide.

We used a supplemental criterion to evaluate the effectiveness of the therapy. This took the form of a study of the average survival among the patients under observation as a function of the particular direct results of the therapy indicated (table 2). We should note that the survival of another 27 patients with morphologically confirmed lung cancer but who received radiotherapy only (the control group) was 5.6 months.

[1]Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, and the P. A. Gertsen Moscow Oncological Institute, Russian Soviet Federated Socialist Republic Ministry of Health.

[2]5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

[3]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

**Reprint requests to:* Dr. A. I. Ruderman, Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, 6 Kashirskoye Chaussee, Moscow, USSR.

TABLE 1.—Response to therapy

Treatment	Total No. of patients	Response		
		Objective improvement	Subjective improvement	Progression
Radiotherapy + 5-fluorouracil	23	15 (65%)	6 (26%)	2 (9%)
Radiotherapy + cyclophosphamide	77	55 (71%)	15 (20%)	7 (9%)
	100	70 (70%)	21 (21%)	9 (9%)

TABLE 2.—Average survival according to response

Response	Average survival (mos)
Objective improvement	18.4
Subjective improvement	6.6
Progression or no effect	4.1

TABLE 3.—Average survival according to dose levels

Total dose levels		Average survival (mos)
Drug (g)	Distant gamma therapy (rads)	
< 3.0	< 5000	5.5
> 3.0	> 5000	8.9

As table 2 shows, the longest survival, longer by a factor of 3 than that of patients treated with radiotherapy alone, was in the group of patients who achieved an objective effect from the therapy. The result is that it is possible to make a direct prediction, to a known degree, of patient survival from the direct results of therapy, and this indicates the obvious desirability of using combination therapy in patients with undifferentiated lung cancer.

A comparison between the average survival rates for patients given a large total dose of radiation and a course of a drug shows that the average survival was < 5.5 months when the focal dose was < 5000 rads and the total dose of the drug was < 3.0 g (table 3). Patient survival was increased to 8–9 months when the total absorbed radiation dose was ≥ 5000 rads and the total dose of the drug was > 3.0 g. The other patients lived > 2 years.

Summarizing the treatment, of the 100 patients, 79 lived for 12 months, 18 lived for > 12 months, and three lived for > 2 years. The use of radiotherapy in combination with cyclophosphamide or 5-fluorouracil in the schemes we studied is not significantly effective in the treatment of undifferentiated lung cancer. Because of the biologic peculiarities of the tumor, a search for new effective variants of combination therapy that involved the use of different drugs and the development of optimum therapy schemes is needed.

Results and Prospects of Combined Surgery and Antitumor Chemotherapy for Lung Cancer

A. I. Pirogov* and A. Kh. Trakhtenberg[1]

One means of making surgical treatment of lung cancer more radical is through the use of combined therapy, which has been studied for 14 years at the P. A. Gertsen Scientific Research Oncological Institute in Moscow. For 6 years, the method has been blind selection of patients for surgical treatment combined with either antitumor drugs or radiation therapy.

The surgical treatment of > 1000 lung cancer patients indicates the primary factors which determine the long-term results of surgical and combined treatment: (*a*) the degree of local distribution of the disease, (*b*) the damage by metastases to the regional lymph nodes, (*c*) the histologic structure of the neoplasm, and (*d*) the method of treatment, either surgery alone or surgery combined with drugs or radiotherapy. Whereas the significance of the first three factors is generally known and is not a subject of debate, the prognostic significance of the method of treatment, particularly combined treatment with supplementary radiation or antitumor chemotherapy, needs further study on the basis of collective experience. Other factors, such as sex, age of the patient, duration and manifestation of disease symptoms, and volume of operation, have some prognostic significance, but their influence is not decisive and depends on the primary factors in the prognosis.

The data from the literature on chemotherapy in combination with radical surgery in lung cancer are contradictory. Whereas Kutschera, Schnetzer, Karrer, Donati, and Ribet report an increase in survival time of patients after combined therapy in comparison to surgical treatment alone, Humphrey, Hughes, Higgins, and Galle report no significant advantage for supplementary chemotherapy; Brunner even reports a worsening of results, manifested as increased frequency of disease recurrence. This contradiction in the evaluation of the effectiveness of combined therapy results, to a significant extent, from variations in the methods of selection of patients, the methods of treatment (particularly chemotherapy), and in the final analysis, the overall approach to the conduct of scientific clinical investigations. The results of the studies performed at the P. A. Gertsen Scientific Research Institute for Oncology in Moscow on the treatment of lung cancer indicate some advantages of combined therapy compared to surgical treatment alone, and indicate situations in which additional antitumor treatment does not improve the long-term results. In addition, directions for further studies aimed at improving the treatment of lung cancer patients are suggested by the results of our studies.

Selection of Patients and Methods of Treatment

During the first period of the clinical study, there were no definite guidelines for the use of combined treatment or for the system of patient selection; during the second period, randomization was strictly performed according to defined, periodically changing characteristics, eliminating subjective decisions in the selection of treatment. In evaluating the results of the investigation of homogeneous groups of patients, the determinants were the degree of surgical treatment and the volume of antitumor chemotherapy. The antitumor preparations used were primarily cyclophosphamide and thioTEPA.[2] The former was given iv, with most patients receiving multiple doses of 1 g each (total dose 4–12 g) either pre- or post-operatively. ThioTEPA was given iv or im at a dose of 10–20 mg, with pre- and post-operative treatment with a total dose of 280–400 mg. The operation was performed 2–3 weeks after chemotherapy, which was resumed 2–4 weeks after the operation. Repeated courses of chemotherapy were given every 3 months for the first year and every 6 months during the second post-operative year. Chemotherapy by this method did not increase the severity of the operation or the post-operative period.

[1]Department of Thoracic Oncology, The Herzen Oncological Institute, Moscow, USSR.

**Reprint requests to:* Prof. A. I. Pirogov, Department of Thoracic Oncology, The Herzen Oncological Institute, Moscow, USSR.

[2]Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

ThioTEPA: NSC-6396; CAS reg. No. 52–24–4; phosphine sulfide, tris(1-aziridinyl)-.

Long-Term Results

The influence of the primary prognostic factors on survival time was estimated for all 678 patients who underwent radical surgery between 1959 and 1972 inclusively and who survived for > 1 year after surgical or combined treatment. For the entire group of patients, a 3-year survival was achieved in 39% (228 of 594 patients) and a 5-year survival in 30% (147 of 497 patients). For stages I and II these survival times were 61.3% and 50.6% respectively; for stage III patients 22.0% achieved a 3-year survival and 14.1% achieved a 5-year survival.

The condition of the lymph nodes and the mediastinum was quite significant for the long-term results of treatment. Among the patients without apparent metastases, 57.7% survived for > 3 years and 46.4% for > 5 years, while among those with apparent metastases in the lymph nodes the survival rates were 23.4% and 16.5% respectively.

Better long-term results were achieved with squamous-cell carcinoma, regardless of the other prognostic factors; the 3-year survival rate for patients with this histologic type was 44.5% and the 5-year rate was 36.1%. With adenocarcinoma, survival rates for these times were 37.0% and 20.6%; the prognostic indicators for the first 3 years after treatment were similar to those for squamous-cell carcinoma. The results of treatment of undifferentiated forms of lung cancer were unsatisfactory, with a 3-year survival rate of only 12.9% and a 5-year survival rate of 8.0%.

Overall, 249 patients received surgery only, 265 received combined surgery and chemotherapy, and 164 received combined surgery and radiation therapy. The fewer number of patients in this last group compared with earlier published reports (1) results from the exclusion of patients treated at the clinic of our Institute (62 patients were treated at the hospital of the Institute).

Chemotherapy combined with radical surgery was accompanied by an increase in survival time of patients compared to surgery alone: 30.7% survived 3 years and 23.1% survived 5 years with surgery alone, while the figures after combined treatment were 46.3% and 36.4% respectively. Thus, the increase in the number of patients receiving surgical and combined therapy over the last 3 years (1970–1972) did not change the overall 3- and 5-year patient survival rates.

This overall tendency was not observed for all histologic forms of cancer and varied with the extent of the tumor, the condition of the lymph nodes at the base of the lung and mediastinum, and the method of combined treatment.

In the early stages (I and II), antitumor chemotherapy helps to increase the survival of patients: 59% survive > 5 years as opposed to 41% after surgery alone. With this sort of local process, chemotherapy decreases the mortality during the first 2 years, whereas after surgery alone the greatest number of deaths occur during these first 3 years, essentially defining the 3- and 5-year survival rates.

Chemotherapy produces a statistically reliable improvement in survival of patients without manifest metastases in the regional lymph nodes: 73.7% survival for > 3 years and 55.9% for > 5 years, as opposed to 43.4% and 38.6% following surgery alone. With regional metastases, the positive influence of antitumor chemotherapy is seen only during the first 3 years after the operation.

The variation in the long-term results between surgery alone and combined treatment can be clearly seen with squamous-cell and undifferentiated lung cancer. With dermoid cancer, the 3-year survival rate for combined therapy was 56.8% and the 5-year survival rate was 45%, whereas with surgical treatment alone the survival rates were 34.8% and 28.7% respectively. With undifferentiated lung cancer in stages I and II and no regional metastases, preference should be given to surgery combined with chemotherapy, while with metastases in the mediastinum, conservative radiation-chemotherapy is preferred. After combined treatment using chemotherapy, 16.3% survived 3 years and 15.3% survived 5 years; with surgical treatment alone, the 3-year survival rate was 6.6% and none survived to 5 years.

Analysis of the long-term results as a function of the preparations and methods of combined treatment used, including repeated courses of chemotherapy, indicates that the influence of both thio-TEPA and cyclophosphamide on the effectiveness of treatment is identical; repeated courses (four to six) over 1–2 years facilitate further improvement of the long-term results. However, all of these questions, which are key questions in combined therapy, require further study and objective scientific evaluation.

The data which we have presented on combination surgery and chemotherapy for lung cancer lead to the following basic conclusions. Pre-operative chemotherapy, using one half of the full-course dose, has no significant influence on the long-term results of surgical treatment. With today's operational techniques, pre-operative chemotherapy does not increase the number of serious post-operative complications or the number of deaths, although it does make the first few post-operative days more severe. In addition, after pre-operative chemotherapy, the leukocyte reaction can not be a reliable indicator of

inflammatory and suppurative complications (pneumonia, suppuration of the wound, empyema, etc).

Statistically reliable improvement of the results of surgical treatment was achieved only with postoperative chemotherapy, particularly in the form of repeated courses of treatment, for patients with stages I and II and no regional metastases during all phases of observation. In these cases, in addition to an increase in the 5-year survival rate, the death rate during the first 3 years is significantly reduced. For stage III, statistically reliable improvement of results was achieved only for the first 3 years after the operation.

Improvement of the results of combined treatment was achieved only for squamous-cell and undifferentiated lung cancer and was not reflected significantly in the glandular form of cancer. The use of large single and total doses of the drugs used in combined therapy is not expedient.

The primary conditions for the application of combined treatment and study of its results with the use of blind selection must be homogeneity of groups with respect to the basic factors of prognosis and radicality of the operation. The results of the study performed by our Institute as well as the results presented in the literature indicate that they may serve as a basis for further studies of combined therapy.

REFERENCE

1. PAVLOV A, PIROGOV A, TRACHTENBERG A, ET AL. Results of combination treatment of lung cancer patients: surgery plus radiotherapy and surgery plus chemotherapy. Cancer Chemother Rep Part 3, vol 4(2):133–135, 1973.

Program Information From the Division of Cancer Treatment, National Cancer Institute

Inhibitors of L-Asparagine Synthetase, In Vitro

David A. Cooney,[1,*] John S. Driscoll,[2] Harry A. Milman,[1] H. N. Jayaram,[1] and Ruth D. Davis[1,3]

SUMMARY

A systematic search has been made for inhibitors of L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) from leukemia 5178Y/AR, a rodent neoplasm resistant to the oncolytic enzyme L-asparaginase (EC 3.5.1.1). The classes of chemicals examined in this search included substrate and product analogs, agents capable of reacting with sulfhydryl functions, and a variety of modifiers whose mechanism of interaction with proteins is known. In general, antagonists of L-glutamine and thiol reagents proved to be the most effective inhibitors of L-asparagine synthetase from this tumor source. Within these groups, certain structural prerequisites to inhibition are reported. Attempts to correlate oncolytic potency with enzyme-inhibitory potency were unsuccessful.

[Cancer Treat Rep 60:1493–1557, 1976]

It has been postulated that the acquired or native ability of certain tumor cells to synthesize L-asparagine at a significant rate is responsible for their resistance to the oncolytic action of L-asparaginase (EC 3.5.1.1) (1). From this postulate, it also has been inferred that total inhibition of L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) in a tumor cell ought to render that cell sensitive to L-asparaginase, provided that the importation of L-asparagine can be minimized. It also follows that the effective termination of the biosynthesis of L-asparagine in a neoplastic cell *developing* resistance to L-asparaginase ought to stop the emergence of that refractory state. Verification of these postulates would mean a tremendous enlargement of the therapeutic scope of L-asparaginase. Using the several inhibitors of L-asparagine synthetase known to date however, experimental oncologists have met with little success in transforming resistant cells into sensitive cells. There are several apparent reasons for this failure: (*a*) L-asparagine derived from the diet or from the continuous hydrolysis of structural and functional proteins may be successfully transported to the tumor cell in compartments, such as the erythrocyte (2), which are impermeable to L-asparaginase; (*b*) there may exist alternate routes for the synthesis of L-asparagine not susceptible to blockade by the inhibitors studied to date (3); (*c*) there may exist several orthodox L-asparagine synthetases which are differentially susceptible to inhibition (4); and (*d*) the inhibitors used have not been powerful enough to stop the synthesis of L-asparagine entirely. It is to this last point that the present paper addresses itself.

A systematic examination has been made of a wide variety of compounds as inhibitors of L-asparagine synthetase of leukemia 5178Y made resistant to L-asparaginase (L5178Y/AR) by administration of subcurative doses of the enzyme. Most of the agents were selected because of their resemblance to one of the substrates or products of the reaction catalyzed by L-asparagine synthetase.

Compounds known for their ability to modify certain functional groups of proteins have constituted the bulk of the remaining agents examined in this screen.

[1]Laboratory of Toxicology, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[2]Drug Design and Chemistry Section, Laboratory of Medicinal Chemistry and Biology, DTP, DCT, NCI, Bethesda, Md.

[3]The authors would like to express their gratitude to Linda Klipp for her valuable help in the preparation of the tables, to G. F. Hazard, Frank Quinn, James Shannon, Mary Flather, and Nancita Lomax for their assistance in obtaining structural information on the compounds reported here, and to the Drafting Unit, Medical Arts Section, National Institutes of Health, for their preparation of the illustrations. Special thanks also go to Dr. Francisco Farina, Instituto de Quimica Organica General, Madrid, Spain, for supplying some of the compounds tested in this study.

Reprint requests to: Dr. David A. Cooney, Laboratory of Toxicology, DTP, DCT, NCI, Bldg 37, Rm 5B22, National Institutes of Health, Bethesda, Md 20014.

MATERIALS AND METHODS

Compounds

The compounds used in the present survey were provided by Dr. Harry Wood or Dr. Robert Engle of the Drug Development Branch, DTP, DCT, NCI, Bethesda, Md, or were purchased from commercial sources. They were used without further purification except where indicated. Immediately before use, each agent was dissolved in water and if necessary, the pH was adjusted to 7.0 with NaOH or HCl.

Antitumor Studies

The antitumor data summarized in tables 1–4 were extracted largely from the records of the Screening Section, Drug Evaluation Branch, DTP, DCT, NCI, Bethesda, Md, for whose cooperation the authors are grateful.

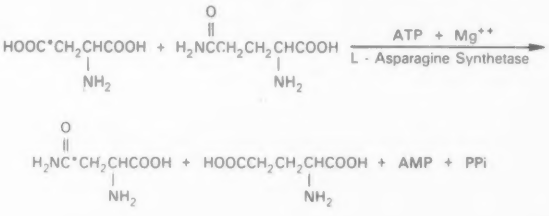
Reagents

L-Glutamate-oxaloacetate transaminase (GOT) (EC 2.5.1.1, specific activity 180 IU/mg of protein) and malate dehydrogenase (MDH) (EC 1.1.1.37, specific activity 1100 IU/mg of protein) were purchased from Boehringer, New York, NY. L-Asparaginase from *E. coli* (EC 3.5.1.1, specific activity 340 IU/mg of protein) was a donation to the NCI from the Merck Institute for Therapeutic Research, Rahway, NJ. L-[4-^{14}C]aspartic acid (specific radioactivity 22.2 microcuries/μmol) was obtained from Biochemical Nuclear Corp, Burbank, Calif, and used without further purification. All other chemicals were of analytic reagent grade.

Procedure for the Measurement of L-Asparagine Synthetase Activity (3)

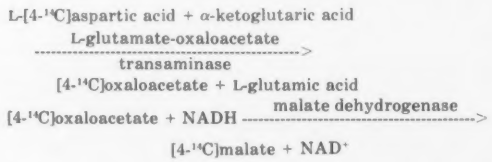
A radioactive "cocktail" was prepared such that each 5 μl contained 1 μmol of ATP, 3 μmols of $MgCl_2$, 0.003 μmol of L-[4-^{14}C]aspartic acid, 2.5 μmols of Tris-HCl buffer (pH 7.6), and either 0.9 μmol of L-glutamine or 2.5 μmols of NH_4Cl.

Incubation: synthesis of L-asparagine.—Ten μl of a 4.5 mM neutralized (pH 7.0) solution of the inhibitor drug was dispensed into six Eppendorf 1500-μl conical centrifuge vessels. To the first three vessels was added 5 μl of the radioactive cocktail containing L-glutamine and to the other three vessels was added 5 μl of the radioactive cocktail containing NH_4Cl. Twenty μl of 0.05 M Tris-HCl buffer, pH 8.4, was added to all vessels. Blank vessels contained 10 μl of water instead of drug solution. Ten μl of 20,000 $\times$ *g* (10 minutes) supernatant of a 1:8 (wt/vol) homogenate of L5178Y/AR in 0.05 M Tris-HCl buffer, pH 8.4, was added to each vessel and the solutions were driven together by a brief (~ 5 seconds) centrifugation at 12,000 $\times$ *g* in the Eppendorf centrifuge. The closed vessels then were placed on the Eppendorf automatic hot-plate at 37°C for 30 minutes after which the reaction was terminated by heating the vessels at 95°C for 5 minutes. In this incubation step, the following reaction takes place:



The presence of an inhibitor of L-asparagine synthetase in the reaction mixture reduced the amount of L-[4-^{14}C]asparagine synthesized as compared to control vessels. After the vessels had cooled, 1 IU of L-asparaginase in a volume of 5 μl was added to the third vessel of each group. This step, which served to hydrolyze L-[4-^{14}C]asparagine to L-[4-^{14}C]aspartic acid, provided an internal control.

"Inactivation" of any unreacted L-[4-^{14}C]aspartic acid.—An "inactivation mix" was prepared by dissolving 5 mg of α-ketoglutaric acid, 10 mg of NADH, 54 IU of L-GOT, and 165 IU of MDH in 0.5 M Tris-HCl buffer, pH 8.4, to a final volume of 250 μl. Ten μl of the resulting mixture was added to each vessel after which the closed vessels were incubated in the Eppendorf hot-plate at 37°C for 30 minutes and then transferred to 95°C for 10 minutes. In this step the following reactions transpired:



At this point, the radioactive components of the experimental vessels were L-[4-^{14}C]asparagine, [4-^{14}C]malate, and approximately 1% of L-[4-^{14}C]aspartic acid which had escaped inactivation. In the control vessels the radioactive components were L-[4-^{14}C]aspartic acid and [4-^{14}C]malate.

Decarboxylation of any unreacted L-[4-^{14}C]aspartic acid.—Fifty μl of a "decarboxylation mix" composed of 10 mg of α-ketoglutaric acid, 50 μl of 70% (wt/vol) $ZnSO_4$, and 18 IU of L-GOT in 10 ml of 0.66 M sodium acetate buffer, pH 5.0, was

added to the cooled vessels. The open vessels were incubated on the Eppendorf hot-plate at 37°C for 60 minutes in the hood. In this step the following reactions occur:

L-[4-^{14}C]aspartic acid + α-ketoglutaric acid
$\xrightarrow[\text{transaminase}]{\text{L-glutamate-oxaloacetate}}$
[4-^{14}C]oxaloacetate + L-glutamic acid

[4-^{14}C]oxaloacetate $\xrightarrow[Zn^{++}]{H^+}$ [^{14}C]O_2 + pyruvate

Now, the radioactive components of the experimental vessels were L-[4-^{14}C]asparagine and [4-^{14}C]malate. The only radioactive component of the control vessels was [4-^{14}C]malate.

Recovery of L-[4-^{14}C]*asparagine.*—To the open vessels containing the "decarboxylation mix," 2 IU of L-asparaginase in a volume of 10 μl was added; immediately thereafter, 5 μl of 40% (wt/vol) KOH was dispensed on the underside of the lid of the vessels (5) and the covers were closed securely. The closed vessels were incubated at 37°C on the Eppendorf hot-plate for 3 hours. In this step the following reactions transpire in the experimental vessels:

L-[4-^{14}C]asparagine $\xrightarrow{\text{L-asparaginase}}$
L-[4-^{14}C]aspartic acid + NH_3

L-[4-^{14}C]aspartic acid + α-ketoglutaric acid
$\xrightarrow[\text{transaminase}]{\text{L-glutamate-oxaloacetate}}$
[4-^{14}C]oxaloacetate + L-glutamic acid

[4-^{14}C]oxaloacetate $\xrightarrow[Zn^{++}]{H^+}$ [^{14}C]O_2 + pyruvate

The [^{14}C]O_2 so generated was collected and counted as previously described (5). The radioactivity recovered in the experimental vessels was compared with that recovered from vessels lacking inhibitor and the percent inhibition calculated accordingly. In certain cases (eg, suramin [A-46])[4] where the putative inhibitor interferred with the analytic technique presented above, L-asparagine was separated from L-aspartic acid electrophoretically on 3 MM paper moistened with 0.1 M sodium phosphate buffer, pH 6.8, using a potential of 3000 volts for 1 hour at 4°C. Ninhydrin spray was used to locate the amino acids which then were excised and quantitated by scintillation spectrometry. Under these conditions L-asparagine remained at the origin. For kinetic analyses, reaction mixtures were constituted and reactions carried out according to the methodology presented in the legends to the appropriate figures.

[4]Bracketed numbers identify the compounds in tables 1–6.

RESULTS AND DISCUSSION

For purposes of presentation, the compounds studied have been aggregated into six groups: (*a*) agents inhibiting L-asparagine synthetase of L5178Y/AR and also exhibiting antitumor activity against one or more standard experimental neoplasms (table 1); (*b*) agents inhibiting L-asparagine synthetase of L5178Y/AR but exhibiting no antitumor activity (table 2); (*c*) agents with antitumor activity but no inhibitory effect against L-asparagine synthetase of L5178Y/AR (table 3); (*d*) agents showing neither antitumor activity nor inhibition of L-asparagine synthetase of L5178Y/AR (table 4); (*e*) inhibitory compounds for which antitumor data are unavailable (table 5); and (*f*) non-inhibitory compounds for which antitumor data are unavailable (table 6). In order to uncover differential effects, the action of each compound has been studied with NH_4Cl as well as with L-glutamine serving as the sources of the amide of the newly synthesized L-asparagine.

For most of the agents examined, only a single molar concentration, 1 mM, of the putative inhibitor was tested in triplicate. In most cases, the results of single experiments are presented; whenever some significant degree of inhibition was observed, additional levels of the inhibitor were studied.

Several conclusions emerge from these tabulations. In the first place, inhibition of L-asparagine synthetase fails absolutely to correlate with the oncolytic activity of known agents. Several excellent inhibitors (maleimide [A-20] and parachloromercuriphenyl sulfonate [A-168], for example) are devoid of antitumor action in our in vivo systems; by the same token many excellent oncolytics are devoid of inhibitory potency towards L-asparagine synthetase. Compounds which possess both antitumor activity and inhibitory activity include the nitrosoureas BCNU [A-6], CCNU [A-8], and methyl-CCNU [A-9], as well as showdomycin [A-5], *cis*-dichlorodiammineplatinum(II) [A-10], and neocarzinostatin [A-11]. In general, the inhibition of L-asparagine synthetase exerted by these oncolytic drugs is rather modest, averaging about 30%.

Other clinically used compounds which lack significant L-asparagine synthetase inhibitory potency are hydroxyurea [A-50], 5-fluorouracil [A-52], duazomycin A [A-56], and azaserine [A-53]. The failure of azaserine, a standard antagonist of L-glutamine, to inhibit this L-glutamine-utilizing enzyme is of considerable theoretical interest.

In light of the failure of the antitumor activity to correlate with the inhibitory activity against L-asparagine synthetase of L5178Y/AR, either in a negative or positive way, the active compounds next

will be discussed briefly on the basis of compound family, structural characteristics, and mechanism of action.

Substrate and Product Analogs

Malonic acid analogs.—Aminomalonic acid [A-157] is the next lower homolog of L-aspartic acid. In vitro analysis revealed that this compound, at a concentration of 1 mM, is a potent inhibitor (about 70%) of the utilization of L-glutamine and also ammonium chloride by L-asparagine synthetase of L5178Y/AR. Horowitz and Meister (4) have reported comparable inhibition of the analogous enzyme from the RADA1 leukemia.[5] The diethyl ester of aminomalonic acid [A-34] is active to a modest degree but the diamide [A-83] is not. It is noteworthy that both the diamide [A-19] and monoamide [A-18] of malonic acid possess modest activity whereas the dithiodiamide [A-84] and mononitrile [A-75] analogs are inactive. The addition of one [A-96] or two [A-284] additional methylene groups to A-19 yielded inactive analogs. Malonic acid itself [A-156b] is without activity.

L-Aspartic acid analogs.—Analogs of L-aspartic acid would be expected to inhibit L-asparagine synthetase by successfully competing with L-aspartic acid or L-asparagine for the appropriate enzymic binding site(s). L-Asparagine itself is known to be a potent product inhibitor of this enzyme (4). Its N^2-acetylated analog [A-279], however, is inactive indicating the importance of a free amino group. The β-hydrazide of aspartic acid [A-35] showed significant inhibition of the utilization of both nitrogen sources by L-asparagine synthetase. Addition of one methylene group [A-106], however, or conversion of the acid to an amide [A-234], abolished activity. The β-hydroxamic acid analog [A-173] was active at 10 mM concentration. Reduction of the carboxyl group of L-isoasparagine [A-293] to a hydroxymethyl group converted an inactive material into an inhibitor of low to moderate potency [A-29]. A similar reduction of the carboxyl group of L-asparagine [A-195d] gave a hydroxymethyl derivative [A-174] with comparable activity. A similar reduction product of L-aspartic acid [A-270] was inactive. Modest activity was observed with a cyclized L-aspartic acid analog [A-178]. Whereas 3-methyl aspartic acid [A-31] possessed a low level of activity, the 3-hydroxy analog [A-99] did not. If the β-carboxylic acid group of L-asparagine is replaced by a phosphonic acid group [A-23], moderate inhibitory activity remains. This was not the case when sulfonic acid [A-209] and *N*-2-chloroethyl sulfamoyl [A-212] groups were substituted. Although essentially all derivatives of succinic acid, including succinimide [A-272], were inactive, *N*-chloro [A-22] and *N*-bromo [A-167] succinimide proved to be good inhibitors. These compounds are powerful halogenating and oxidizing agents. The cyclic *N*-hydroxy derivative [A-21] showed a low level of activity. Of interest is the inhibition produced by oxaloacetate [A-195b]. This compound is the keto acid counterpart of L-aspartic acid and might therefore be expected to behave as a competitive inhibitor. Analysis of double-reciprocal plots of the inhibition produced by oxaloacetate showed that this was the case (fig 1). This finding indirectly suggests that oxaloacetate may be amidated by L-asparagine synthetase to yield α-ketosuccinamic acid [A-278], a known precursor of L-asparagine.[6] In this context, it is noteworthy that malate [A-156d], the hydroxy analog of L-aspartic acid, produced no inhibition even at a concentration of 10 mM.

Simple analogs of L-glutamine.—L-Glutamic acid [A-37] has a low level of inhibitory activity at 10–50 mM concentrations. The hydroxamic acid [A-169] and β-aminoglutamic acid [A-170] are somewhat active but the addition of one more methylene unit to the later compound [A-214] nullifies inhibition. L-Glutamic acid esters [A-125, A-218, A-285] and *N*-substituted L-glutamines [A-106, A-116, A-221] are inactive. Dehydration of L-glutamine to its nitrile [A-232] or formation of the N^2-urea derivative [A-198] gives inactive materials. As in the case of the L-aspartic acid analog, replacement of the γ-carboxyl group in L-glutamic acid with the phosphonic acid function [A-24] gave an inhibitory compound. Use of the sulfoximine group in the same manner gave a non-active compound [A-113].

Diazomethyl ketone analogs of L-glutamine (fig 2).—The chemically reactive diazomethyl ketones azotomycin [A-1], DON [A-2], and DONV [A-4], as well as the related chloromethyl compound CONV [A-3], all inhibited L-glutamine utilization by L-asparagine synthetase from leukemia 5178Y/AR with no observable inhibition of ammonia utiliza-

[5]These authors were unable to demonstrate that aminomalonic acid was amidated to aminomalonamic acid by L-asparagine synthetase from RADA1 leukemia. In our studies, too, the analogous synthetase from L5178Y/AR did not appear to amidate aminomalonic acid in an assay which measures the L-aspartate- or aminomalonate-dependent breakdown of L-[U-^{14}C]glutamine to L-[U-^{14}C]glutamic acid in the presence of ATP and magnesium chloride.

[6]It should be noted, however, that transaminases in the crude enzyme preparation used for these studies might have transaminated oxaloacetate with L-glutamic acid arising from the amidohydrolysis of L-glutamine, a concomitant reagent. The product of such a transamination is L-[4-^{12}C]aspartic acid which would give rise to apparently competitive inhibition by diluting the specific activity of the L-[4-^{14}C]aspartic acid used as a reagent. The exact nature of the inhibition exerted by oxaloacetate must, therefore, await further studies with a purified enzyme preparation.

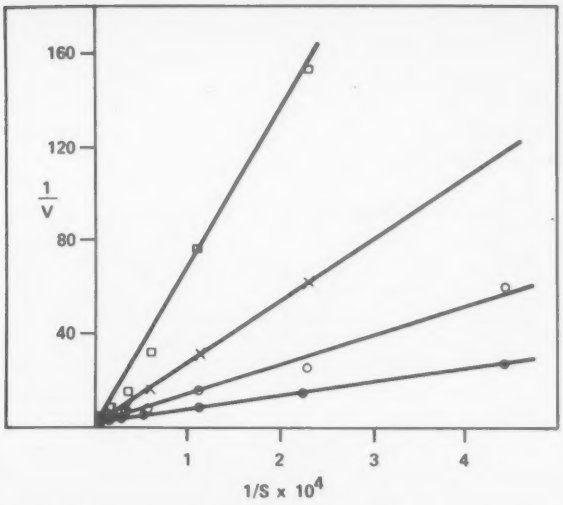


FIGURE 1.—Apparent competition of oxaloacetic acid with L-aspartic acid for L-asparagine synthetase of L5178Y/AR. 5 μl of pH 7.0 oxaloacetic acid solution (3, 9, or 18 mM) or 5 μl of water was admixed in a 1500-μl Eppendorf conical centrifuge vessel with 5 μl of a radioactive "cocktail" composed of 0.2 μmol of L-glutamine, 0.1 μmol of ATP, 0.25 μmol of $MgCl_2$, 2.5 μmol of Tris-HCl buffer (pH 7.6), and either 0.0384, 0.0192, 0.0096, 0.0048, 0.0024, 0.0012, or 0.0006 μmol of L-[4-^{14}C]aspartic acid. 5 μl of 12,000 × *g* (3 mins) supernatant of L5178Y/AR homogenate (1:4; wt/vol in 0.05 M Tris-HCl buffer, pH 8.4) was then added to each vessel and the solutions were driven together by a quick (approximately 5 secs) centrifugation at 12,000 × *g*. The vessels then were incubated at 37°C for 30 mins after which they were heated at 95°C for 5 mins. Subsequent steps in the assay are as outlined in the Materials and Methods section. All points are the means of triplicate vessels.

tion. DONV, a reactive analog of L-asparagine, is a more feeble inhibitor (27% at 1 mM). DON, a prototypical diazo analog of L-glutamine which differs from DONV only by an additional methylene group, exerted 82% inhibition at the same concentration. These findings are in agreement with the observation made with the homologous enzyme from KB cells (6) and from the 6C3HED lymphosarcoma of mice (7). The diazomethyl function is not always sufficient for activity, however, as azaserine [A-53] is inactive; also, *N*-acetylation of DON, to produce duazomycin A [A-56], abolished activity.

Isoxazole derivatives.—The stereochemical resemblance of L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid [A-12] to L-glutamine, the amide precursor of L-asparagine, prompted an examination of the agent as an inhibitor of L-asparagine synthetase. In vitro, this drug was found to produce potent inhibition (approximately 95%) of L-glutamine utilization by the enzyme; no inhibition of ammonia utilization was produced under these circumstances. Noteworthy too is the finding that this agent exhibits a high order of in vivo activity against leukemia L1210. Most other isoxazole analogs were found to exert no inhibition of L-asparagine synthetase. However, ibotenic acid [A-48a], a compound of fungal origin, was significantly inhibitory. In this case, the compound inhibited the utilization of both L-glutamine and ammonia and so is unlikely to be acting by the same mechanism as [A-12].

Analogs of ammonia.—Although L-asparagine synthetase exhibits a K_m for ammonia that is roughly 20 times higher than its K_m for L-glutamine, at saturation the V_{max} achieved by the enzyme with ammonia is equivalent to the V_{max} with L-glutamine. A search was therefore conducted for analogs of ammonia.[7] Hydrazine [A-65], methylamine hydrochloride [A-156a], and hydroxylamine [A-14] all were able to inhibit the synthesis of L-asparagine from L-aspartic acid and ammonia, but very high concentrations (10 mM) of these agents were required for the demonstration of this effect. Whether such small amines can substitute for NH_3 in the synthesis of L-

[7] At the pH optimum of L-asparagine synthetase, NH_3 as opposed to NH_4^+ is the preponderant chemical species.

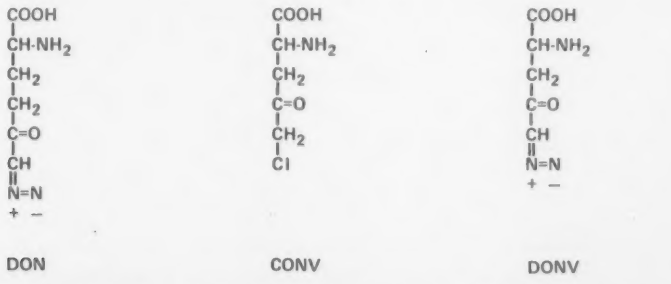


FIGURE 2.—Structures of DON, DONV, and CONV.

asparagine-like compounds, as they can in the L-glutamine synthetase reaction (8), requires further study.

Analogs of adenosine-5′-triphosphate.—A small number of analogs of ATP, the ordinary energy source for the synthesis of L-asparagine, were examined in the hope of uncovering a strong competitive inhibitor. Of these, etheno ATP [A-289], β-γ-methylene ATP [A-290], 3′,5′cyclic AMP [A-150], UTP [A-250], ITP [A-249], CTP [A-246], TTP [A-251], NADH [A-244], and NADP [A-248] exerted no inhibition of enzyme, while NAD [A-184], NMN [A-186], and ADP [A-185] produced marginal effects. It is noteworthy that dATP [A-291] and GTP [A-292] were not inhibitory to the enzyme; in separate studies, however, these nucleotides were found to substitute for ATP as energy sources.

Pyrophosphate analogs.—In the synthesis of L-asparagine, ATP is split pyrophosphorylytically to AMP and pyrophosphate (3). Although the addition of inorganic pyrophosphatase to reaction mixtures does not increase the apparent rate of L-asparagine synthesis, it is possible to show that sodium pyrophosphate [A-195c] can behave as a product inhibitor.* Therefore, a small number of analogs of pyrophosphate have been examined as candidate inhibitors. Neither ethylene diphosphonic acid [A-80] nor methylenebisphosphonic acid [A-261] were inhibitory at a concentration of 1 mM.

Metal ions and other inorganic cofactors.—Magnesium ions are the ordinary metal cofactors in the synthesis of L-asparagine (4). An examination of a number of ions neighboring magnesium in the periodic table revealed that zinc [A-183] (in the form of zinc sulfate) virtually eradicated the synthesis of L-asparagine by the tumoral enzyme, and that calcium chloride [A-187] was also significantly inhibitory irrespective of whether L-glutamine or ammonia was a substrate. Cobaltous chloride [A-188] however, preferentially impaired the synthesis of L-asparagine from ammonia while inhibiting the utilization of L-glutamine only to a minor degree. This selective effect is reminiscent of the inhibitory activity of mucochloric acid [A-26] (*see* protein modifiers). The rather prominent inhibition exerted by zinc and calcium ions may be of physiologic importance in the modulation of the synthesis of L-asparagine in vivo. In this connection it is relevant to review the molar concentration of these metals prevailing in vivo. Zinc, calcium, and cobalt are present in human plasma at concentrations of 4.5×10^{-5}M, 2.5×10^{-4}M, and 1.6×10^{-6}M respectively; in human pancreas, zinc and calcium are present at concentrations of 3×10^{-4}M and 3.5×10^{-3}M (9) Thus, there is reason to suggest that these ions may inhibit the synthesis of L-asparagine in vivo, although compartmentation and ionization probably lower their effective cytoplasmic concentration below the levels cited. Obviously, such a control mechanism merits further study. Horowitz and Meister (4) reported that the chloride ion interacts in a prominent way with L-asparagine synthetase from RADA1 leukemia, activating the enzyme in the process. For this reason, a number of halides have been examined versus the enzyme from L5178Y/AR in the anticipation that these ions might interrupt such an interaction. However, neither fluoride [A-256], iodide [A-255], nor bromide [A-257] inhibited the enzyme under the conditions of these experiments.

Protein Modifiers (Sulfhydryl Reagents)

Although DON and DONV are analogs of L-glutamine and of L-asparagine, there is also evidence that these agents inhibit by interacting with sulfhydryl functions at the L-glutamine site of L-asparagine synthetase; thus, a molar excess of dithiothreitol added to an enzyme preparation in advance of the diazoketones shields the enzyme from inhibition (10–12). This finding prompted an examination of the inhibitory behavior of other known sulfhydryl reagents.

Ethacrynic acid.—Ethacrynic acid [A-45], a clinically useful diuretic, has been tested as an inhibitor of L-asparagine synthetase because of its well-studied ability to react with sulfhydryl functions reversibly. In vitro, ethacrynic acid exerted significant inhibition of the utilization of L-glutamine and NH_4Cl by the enzyme (46% and 63% respectively at 1 mM drug concentration). Several analogs of ethacrynic acid were also effective inhibitors, most notably 2-(1-carboxymethoxy-4-chloro-2-naphthyl)-3-6-diethyl-6-(1-carboxymethoxy-4-chloro-2-naphthoyl)-5,6-dihydro-[4*H*]-pyran [A-195a].

Mapharsen.—Mapharsen [A-44], an arsenical and one of the earliest weapons in the chemotherapeutic armamentarium, was also found to inhibit both L-glutamine and ammonia utilization (53% and 79% respectively at 1 mM drug concentration). The nonselectivity of this agent as well as of ethacrynic acid stands in contrast to the selective inhibition of L-glutamine utilization achieved by DON, CONV, and DONV. Thus, whereas all of the aforementioned

*This effect can be seen in table 5; a possible explanation for it is that pyrophosphate is chelating magnesium ions and thereby lowering the effective concentration of the ATP-Mg^{++} complex. It is noteworthy that Horowitz and Meister found that pyrophosphate was a considerably more potent inhibitor of L-asparagine synthetase from RADA1 leukemic cells than of the analogous enzyme from L5178Y/AR cells; thus, 2 mM pyrophosphate inhibited 50% (table 5 of ref 4) and 0.5 mM pyrophosphate inhibited ~60% (fig 9 of ref 4). In these studies pyrophosphate behaved as a competitive inhibitor of the enzyme versus ATP.

drugs may be acting via sulfhydryl blockade, they cannot all be acting wholly at the same sites.

Mucobromic and mucochloric acids.—The structures of these two halogenated organic acids are sterically similar to that of L-aspartic acid. It was for this reason that they were initially examined as candidate inhibitors of L-asparagine synthetase. The pattern of inhibition produced by both agents versus the enzyme from L5178Y/AR proved to be distinctive; the utilization of L-glutamine was only modestly depressed by the inhibitors at 1 mM, but the utilization of ammonia was sharply curtailed (about 70%). These effects are, of course, the reverse of those produced by the diazoketones discussed above. Subsequent studies have demonstrated that the preferential inhibition of the utilization of ammonia is pH dependent and that an alkaline pH reveals it best. An analysis of the kinetics of the inhibition has shown that mucochloric acid [A-26] is a noncompetitive inhibitor with L-aspartic acid and ammonia but an apparently competitive inhibitor with L-glutamine as a substrate.[9] Moreover, both L-glutamine and dithiothreitol were able to prevent the inhibition exerted by both of these halogenated acids to a significant degree. It therefore seems warranted to conclude that mucochloric acid and mucobromic acid [A-25] are reacting with critical sulfhydryls at the L-glutamine and ammonia sites of L-asparagine synthetase and are not inhibitors by virtue of their structural similarity to L-aspartic acid. The furanones [A-175, A-176, A-195] which can be viewed as cyclized derivatives of mucobromic and mucochloric acids were, as a class, active inhibitors. A halogen attached to the double bond appears essential for such activity [A-273]. A nitrogen analog [A-204] was inactive.

Maleimide and its precursors.—If at any point in the course of its synthesis, transamination, or hydrolysis, L-asparagine were to assume a cyclic configuration, the resulting intermediate would bear a strong resemblance to maleimide, a powerful sulfhydryl reagent. It therefore was advisable to test this drug and its congeners as possible inhibitors of L-asparagine synthetase. Showdomycin [A-5] and maleimide [A-20] as well as *N*-ethyl maleimide [A-48b] exerted powerful inhibition of L-glutamine utilization by L-asparagine synthetase of L5178Y/AR in vitro at 1 mM; lesser inhibition of the utilization of ammonia, the alternate substrate, was also observed. Mercaptoethanol and dithiothreitol in molar excess protect against the inhibition exerted by maleimide. The addition of a large, lipophilic electron-withdrawing group attached to the ring nitrogen, as in the case of iodophenylmaleimide [A-181], completely eliminated any inhibitory activity against both substrates. Likewise, substitution on the double bond and the 2 and 3 positions (as in the case of 2-ethoxy-3-phenyl maleimide [A-204]) completely eradicated inhibition. In studying the inhibitory activity exerted by straight-chain precursors and cyclic analogs of maleimide, it was found that only the anthracene-substituted maleamic acid derivatives (1-NH_2 [A-166] and 2-NH_2 [A-165] anthracene maleamic acids) exerted any inhibitory activity against L-asparagine synthetase of L5178Y/AR in vitro (approximately 50% inhibition of L-glutamine and ammonia utilization at 1 mM). Neither succinimide [A-272], succinamide [A-96], succinamic acid [A-98], nor maleic acid [A-156c] and its derivatives exerted any inhibitory activity. The addition of a halogen, however, on the nitrogen of succinimide (eg, *N*-bromosuccinimide [A-167]) was sufficient to exert nearly 100% inhibition of the utilization of both substrates by L-asparagine synthetase of L5178Y/AR at 1mM concentration. Dithiothreitol completely prevented this inhibition, probably by reducing A-167. Therefore, for optimum inhibitory potency, the nitrogen of maleimide may be unsubstituted or substituted with a short aliphatic chain (eg, *N*-ethyl maleimide [A-48b]). While the aromatic dimaleimide [A-277] was inactive, its maleic anhydride analog [A-179] had a low level of activity.

Diamide.—Diamide [A-38] has found experimental use as a reagent for the oxidation of intracellular glutathione of erythrocytes (13). The sulfhydryls of L-asparagine synthetase were similarly susceptible, with 1 mM diamide achieving very powerful inhibition of the synthesis of L-asparagine from both nitrogenous precursors. In view of the ability of this reagent to penetrate cells, attempts to use it for the inhibition of L-asparagine synthetase in vivo will be of interest.

Mercurials.—Mercurials are among the oldest, most powerful, and most toxic of the sulfhydryl reagents. In the present enzymatic screen, *p*-(chloromercury)-phenylsulfonic acid [A-168] showed itself to be an excellent inhibitor of the enzyme from L5178Y/AR.

Other Modifiers

It is of considerable interest that urea [A-15] and certain of the nitrosoureas did inhibit the target enzyme to a modest degree. Inasmuch as the molarity of urea used for these experiments (1–10 mM) was insufficient to have altered the tertiary structure of L-asparagine synthetase, it is not likely to be operating by this mechanism. The activity of the nitrosoureas can probably be ascribed to their ability to carbamylate the ϵ-amino functionality of criti-

[9]Cooney DA, Milman HA, Jayaram HN, et al. Unpublished observations.

cal L-lysine residues on the enzyme. The activities of A-3, A-16, A-17, and A-40 may also be related to their alkylating capacity. That heparin [A-177] could inhibit L-asparagine synthetase to a prominent degree is of considerable interest, inasmuch as this anticoagulant is present normally in vivo and might, therefore, play a role in regulating the rate of biosynthesis of L-asparagine. Mechanistically speaking, it seems unlikely that heparin is interacting with the enzyme through electrostatic forces inasmuch as both macromolecules bear a net negative charge at physiologic pH; nevertheless, the possibility that L-asparagine synthetase has a "run" of basic amino acid residues in the vicinity of its active site cannot be ruled out.

CONCLUSION

Although many interesting leads have been uncovered in the present screen, no compound meeting the requirements set forth in the introduction has been discovered thus far. There is need for an agent achieving total or nearly total and irreversible inhibition of the synthesis of L-asparagine from both of its nitrogen-donating substrates, at concentrations of 1×10^{-3}M or preferably lower, if resistance to L-asparaginase is to be prevented and/or reversed.

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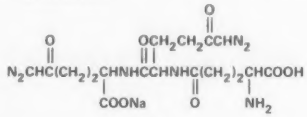
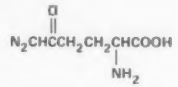
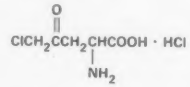
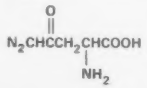
TABLES 1–6.—The oncolytic activity of the various agents was determined according to the protocols of the Drug Research and Development Program, DCT, NCI (14). In the test systems using survival as the end point, antitumor activity was computed as %T/C [T/C = (treated survival ÷ control survival) × 100%]. A test with a T/C ≥ 125% is defined as an active test. In these test systems, one measure of toxicity is the number of survivors on Day 5. In the test systems using tumor weight inhibition as the criterion for activity, a T/C value ≤ 42% (58% tumor inhibition) is necessary for an agent to be considered active. Survivors in these systems, in general, are recorded on the last day of the test. The doses of drugs given represent range of drug doses used and do not necessarily reflect the optimal therapeutic dose. "Z" indicates treatment until death of animals. *Designation of Tumors.*—The following abbreviations have been used to designate the tumor systems used: S180 = Sarcoma 180; L1210 = L1210 lymphoid leukemia; L5178Y = L5178Y lymphatic leukemia; P388 = P388 lymphocytic leukemia; LL = Lewis lung carcinoma; P1534 = P1534 leukemia. L-Asparagine synthetase was measured according to the methodology given in the Materials and Methods section. Percent inhibition was calculated by comparison to control vessels receiving no inhibitor.

TABLE 1. Compounds Possessing Antitumor Activity and Inhibiting L-Asparagine Synthetase from L5178Y/AR, In Vitro

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Amide Donors Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
1	Glutamine, N-{1-[(1-carboxy-5-diazo-4-oxopentyl)-carbamyl[-5-diazo-4-oxopentyl}-, sodium salt (NSC-56,654) AZOTOMYCIN 	1.0	50	0	L1210	+	12.5 x 1 100.0 x 1	10/10 10/10
					L5178Y	+	20.0 x 3 80.0 x 3	6/6 6/6
					P388	+	5.0 x 5 20.0 x 5	6/6 6/6
2	Norleucine, 6-diazo-5-oxo-, L- (NSC-7,365) DON 	0.1	82	0	Gardner 6C3HED lympho-sarcoma	+	0.03 x 10 0.20 x 10	6/6 6/6
					L1210	+	50.0 x 2 400.0 x 2	10/10 7/10
					P388	+	2.5 x 5 20.0 x 5	6/6 5/6
					Walker carcino-sarcoma 256	+	0.06 x 4 1.0 x 4	6/6 3/6
3	Levulinic acid, 2-amino-5-chloro-, hydrochloride, L- (NSC-124,412) CONV 	0.1 0.5	55 72	0 0	L1210	+	18.8 x 9 75.0 x 9	6/6 5/6
		1.0	83	0	L5178Y	-	3.13 x 5 50.0 x 5	6/6 1/6
4	Norvaline, 5-diazo-4-oxo-, L- (NSC-117,613) DONV 	1.0	27	0	L1210	+	50.0 x 9 800.0 x 9	6/6 6/6
					P388	-	100.0 x 3 400.0 x 3	6/6 6/6

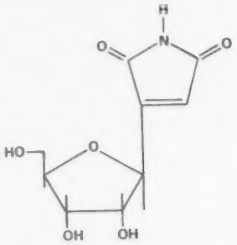
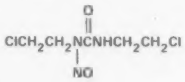
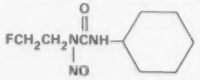
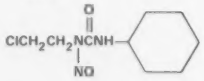
5	1H-Pyrrole-2,5-dione, 3-β-D-ribofuranosyl- (NSC-93,047) SHOWDOMYCIN 	0.5 1.0	56 78	58 89	L1210	-	5.0 x 9 55.0 x 2	6/6 0/6
					L5178Y	-	2.0 x 10 32.0 x 5	6/6 0/6
					P388	+	0.20 x 10 32.0 x 10	6/6 0/6
					S180	-	3.0 x 7 50.0 x 7	6/6 0/6
					Walker carcino-sarcoma 256	-	6.0 x 4 25.0 x 4	6/6 3/6
6	1,3-Bis(2-chloroethyl)-1-nitrosourea (NSC-409,962) BCNU 	1.0	30	48	S180	+	1.5 x 7 24.0 x 7	6/6 2/6
					L1210	+	17.0 x 1 88.0 x 1	10/10 0/10
					P388	+	3.13 x 10 25.0 x 10	6/6 6/6
					L5178Y	+	6.25 x 10 50.0 x 10	6/6 6/6
					Gardner 6C3HED lympho-sarcoma	+	12.5 x 10 50.0 x 10	6/6 2/6
7	Urea, 3-cyclohexyl-1-(2-fluoro-ethyl)-1-nitroso- (NSC-87,974) 	1.0	26	34	L1210	+	6.0 x 1 96.0 x 1	10/10 0/10
					P388	+	6.25 x 3 100.0 x 3	6/6 0/6
8	Urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso- (NSC-79,037) CCNU 	1.0	31	35	L1210	+	19.4 x 1 200.0 x 1	8/8 8/10
					Walker carcino-sarcoma 256	+	3.0 x 4 100.0 x 4	6/6 0/6
					B16 melano-carcinoma	+	8.0 x 9 64.0 x 9	10/10 8/10

TABLE 1. (Continued)

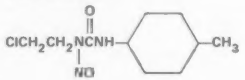
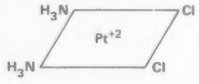
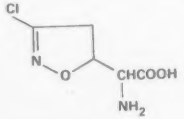
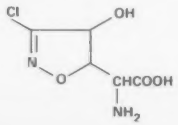
A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
9	Urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-, trans- (NSC-95,441) METHYL-CCNU 	1.0	23	42	B16 melano-carcinoma	+	1.90 x 1 60.0 x 1	6/6 6/6
					L1210	+	15.0 x 1 75.0 x 1	6/6 0/6
					LL	+	3.12 x 1 100.0 x 1	9/10 3/10
10	Platinum, diamminedichloro-, cis- (NSC-119,875) 	1.0 2.0	28 27	39 31	L1210	+	0.3 x 9 9.6 x 9	10/10 1/10
		5.0	28	44	P388	+	0.30 x 10 9.6 x 10	10/10 10/10
					Walker carcino-sarcoma 256	+	0.31 x 4 20.0 x 4	6/6 0/6
11	Neocarzinostatin, crystalline antibiotic (NSC-157,365) *(Primary Structure Available in: Science 178: 875-876, 1972)* Protein	0.005 1.0 5.0	0 18 53	0 23 48	L1210	+	0.06 x 9 8.0 x 9	10/10 0/10
12	L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid (NSC-163,501) 	1.0	85	0	L1210	+	1.0 x 9 125.0 x 9	10/10 5/10
					LL	-	1.0 x 13 32.0 x 13	8/8 4/8
					P388	+	3.12 x 3 100.0 x 3	8/8 8/8
13	L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-4-hydroxy-5-isoxazoleacetic acid (NSC-176,324) 	1.0	79	0	L1210	+	3.12 x 9 150.0 x 9	10/10 6/6
					P388	+	12.5 x 9 250.0 x 9	6/6 6/6
					LL	-	8.0 x 13 256.0 x 13	8/8 8/8

TABLE 2. Compounds Lacking Antitumor Activity But Inhibiting L-Asparagine Synthetase From L5178Y/AR, In Vitro

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
14	Hydroxylamine, hydrochloride (NSC-26250)	1.0	23	28	Ca755	-	22.5 x 11	9/10
	$H_2NOH \cdot HCl$				L1210	-	62.5 x Z 500.0 x Z	6/6 0/6
					S180	-	12.5 x 14 250.0 x 14	6/6 0/6
15	Urea (NSC-34375)	1.0	28	16	Ca755	-	400.0 x 11	10/10
	H_2NCNH_2 (O)	10.0	28	27	L1210	-	400.0 x Z	6/6
		100.0	21	36	S180	-	500.0 x 7	6/6
16	Propanoic acid, 3-bromo- (NSC-2638)	1.0	0	0	Walker carcino-sarcoma 256	-	100.0 x 4	6/6
		5.0	14	0			400.0 x 4	0/6
	$BrCH_2CH_2COOH$	10.0	27	0				
17	Propionitrile, 3-chloro- (NSC-2588)	1.0	0	29	Ca755	-	25.0 x 11 50.0 x 11	1/10 1/10
	$ClCH_2CH_2CN$				L1210	-	100.0 x Z	0/6
					S180	-	50.0 x 14 250.0 x 14	10/10 0/10
18	Malonamic acid (NSC-1812)	1.0	0	0	Ca755	-	250.0 x 11	6/7
	H_2NCCH_2COOH (O)	5.0	14	0	L1210	-	500.0 x Z	6/6
		10.0	29	16	S180	-	50.0 x 14 250.0 x 14	8/10 0/10
					Walker carcino-sarcoma 256	-	400.0 x 4	6/6
19	Malonamide (NSC-2134)	1.0	0	23	Ca755	-	175.0 x 11	6/6
	$H_2NCCH_2CNH_2$ (O, O)	5.0	0	23	L1210	-	350.0 x Z	6/6
					Walker carcino-sarcoma 256	-	400.0 x 7	6/6

TABLE 2. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
20	Maleimide (NSC-13684)	1.0	87	42	L1210	-	5.0 x 1	6/6
							20.0 x 1	0/6
					P388	-	0.93 x 9	6/6
							3.74 x 9	5/6
					S180	-	85.0 x 14	0/6
21	Succinimide, N-hydroxy- (NSC-74,335)	1.0	16	20	L1210	-	50.0 x Z	6/6
		10.0	17	13			400.0 x Z	6/6
22	Succinimide, N-chloro- (NSC-8,748)	0.1	14	27	Ca755	-	18.0 x 11	10/10
		1.0	92	75	L1210	-	20.0 x Z	5/6
					S180	-	10.5 x 14	6/6
							250.0 x 14	0/6
23	Alanine, 3-phosphono- (NSC-30,078)	1.0	31	29	L1210	-	50.0 x Z	6/6
					S180	-	30.0 x 14	6/6
							250.0 x 14	3/6
24	Butyric acid, 2-amino-4-phosphono- (NSC-30,079)	1.0	37	46	Ca755	-	225.0 x 11	5/6
							450.0 x 11	4/6
					L1210	-	450.0 x Z	6/6
					S180	-	250.0 x 14	6/6
25	(2,3-Dibromo-3-formylacrylic acid) (NSC-12,426) MUCOBROMIC ACID	0.5	0	40	L1210	-	3.0 x Z	7/10
		1.0	25	75			65.0 x Z	2/10
					S180	-	50.0 x 14	0/6

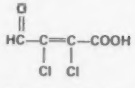
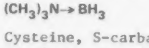
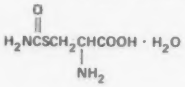
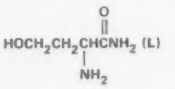
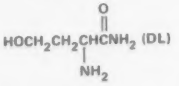
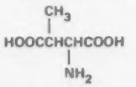
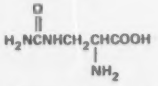
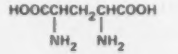
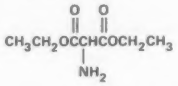
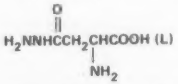
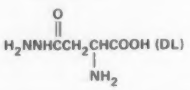
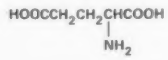
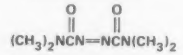
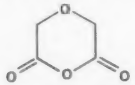
26	(Dichloro-malealdehydic acid) (NSC-15,905) MUCOCHLORIC ACID 	0.1 0.5 1.0 10.0	13 15 24 96	23 63 60 90	Ca755	-	2.25 x 11 18.0 x 11	10/10 9/10
27	Trimethylamine borane (1:1) (NSC-145,941) 	1.0	0	26	L1210	-	3.13 x 1 400.0 x 1	10/10 1/10
28	Cysteine, S-carbamate, monohydrate, L- (NSC-102,498) 	1.0 10.0	47 62	33 51	L1210	-	200.0 x 15 400.0 x 15	5/6 3/6
					Walker carcinosarcoma 256	-	200.0 x 4	6/6
29	Butyramide, 2-amino-4-hydroxy-, monohydrochloride, L- (NSC-138,784) 	1.0 5.0	12 23	20 20	L1210	-	75.0 x 9 300.0 x 9	6/6 6/6
					L5178Y	-	100.0 x 3 400.0 x 3	/6 6/6
30	Butyramide, 2-amino-4-hydroxy-, monohydrochloride, DL- (NSC-138,785) 	1.0	22	24	L1210	-	75.0 x 9 300.0 x 9	6/6 6/6
					L5178Y	-	100.0 x 10 400.0 x 10	6/6 6/6
31	DL-Aspartic acid, 3-methyl- (NSC-45,365) 	1.0	25	25	L1210	-	100.0 x 1 400.0 x 1	6/6 6/6
					S180	-	250.0 x 14	6/6
32	Propionic acid, 2-amino-3-ureido-, L- (NSC-407,273) ALBIZZIN 	10.0	77	62	Ca755	-	160.0 x 11	10/10
					S180	-	80.0 x 7	6/6
33	Glutaric acid, 2,4-diamino- 	1.0 10.0	0 43	0 0	L1210	-	400.0 x 9	6/6

TABLE 2. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
34	Malonic acid, amino-, diethyl ester (NSC-121,992) 	1.0	33	15	L1210	-	400.0 x 9	6/6
35	Aspartic acid, 4-hydrazide, hemihydrate, L- (NSC-130,683) 	1.0	57	60	L1210	-	400.0 x 1	6/6
36	Aspartic acid, 4-hydrazide, DL- (NSC-19,427) 	1.0 10.0	0 57	0 63	Ca755	-	125.0 x 11	10/10
37	L-Glutamic acid, hydrochloride (NSC-9,239) 	10.0 50.0	14 41	0 0	Ca775 L1210	- -	225.0 x 11 450.0 x 11 125.0 x Z 500.0 x Z	9/10 0/10 6/6 1/6
38	Formamide, 1,1'-azobis[N,N-dimethyl- DIAMIDE (NSC-143,013) 	0.01 0.1 1.0	18 50 87	0 30 89	L5178Y	-	12.5 x 5 100.0 x 5	6/6 6/6
39	1,4-dioxane-2,6-dione 	1.0 10.0	12 8	23 25	L1210 S180	- -	50.0 x Z 60.0 x 7	6/6 6/6
40	Phosphine sulfide, tris (1-aziridinyl)- (NSC-6,396) ThioTEPA	1.0	19	26	Gardner 6C3HED lymphosarcoma	-	0.75 x 10 8.0 x 10	6/6 6/6

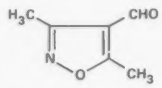
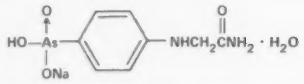
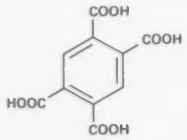
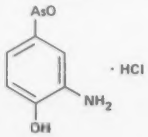
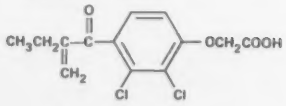
41	4-Isoxazolecarboxaldehyde, 3,5-dimethyl- (NSC-151,753) 	1.0	23	30	L1210	-	400.0 x 3 100.0 x 3	2/6 6/6
42	Arsanilic acid, N-(carbamoylmethyl)-, sodium salt, hydrate (NSC-2,050) TRYPARSAMIDE 	1.0	0	29	S180	-	250.0 x 14	6/6
					Walker carcino-sarcoma 256	-	400.0 x 3	6/6
43	Pyromellitic acid (NSC-6,369) 	1.0	17	17	Ca755	-	250.0 x 11	6/6
		2.5	12	27	L1210	-	500.0 x 2	7/7
					S180	-	250.0 x 11	6/6
					L5178Y	-	177.0 x 5 600.0 x 5	7/7 6/7
44	Phenol, 2-amino-4-arsenoso-, hydrochloride (NSC-3,087) MAPHARSEN 	0.1	0	0				
		1.0	53	79	Ca755	-	9.0 x 11	10/10
					L1210	-	38.0 x 9 75.0 x 1 400.0 x 1	6/6 0/6 0/6
45	Acetic acid, [2,3-dichloro-4-(2-methylene-butyryl)phenoxy]- (NSC-85,791) ETHACRYNIC ACID 	1.0	46	63	S-91 Cloudman melanoma	-	100.0 x 11	0/10
		2.5	76	84			400.0 x 11	0/10

TABLE 2. (Continued)

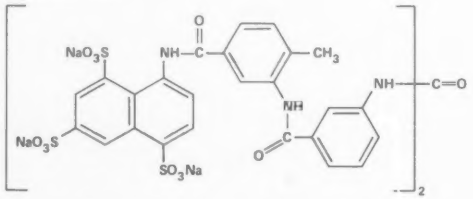
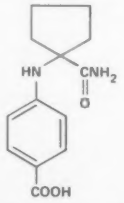
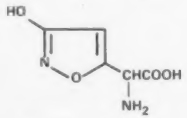
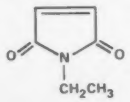
A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
46	1,3,5-Naphthalenetrisulfonic acid, 8,8' [ureylene-bis[m-phenylenecarbonylimino (4-methyl-m-phenylene)carbonylimino]] di-, hexasodium salt (NSC-34,936) SURAMIN SALT	1.0	0	6	S180	-	15.0 x 14	5/6
		5.0	80	100			250.0 x 14	0/6
					L1210	-	50.0 x 15	6/6
							400.0 x 15	2/6
								
47	Benzoic acid, p(1-carbamoylcyclopentylamino)- (NSC-43,110)	1.0	23	32	Ca755	-	400.0 x 11	9/10
					L1210	-	400.0 x Z	6/6
					S180	-	250.0 x 14	5/6
48	Decaborane (NSC-39,828) $B_{10}H_{14}$		38	40	L1210	-	200.0 x Z	6/6
					S180	-	125.0 x 14	5/6
48a	Ibotenic acid (NSC-204,850)	1.0	26	21	P388	-	1.0 x 9	3/3
		10.0	70	65			0.25 x 9	3/3
								
48b	N-Ethyl maleimide (NSC-7,638)	1.0	90	48	L1210	-	4.0 x Z	0/6
							2.0 x Z	4/6
					S180	-	42.0 x 14	0/6
							2.0 x 14	6/6

TABLE 3. Compounds Possessing Antitumor Activity but Negligible Inhibition of L-Asparagine Synthetase from L5178Y/AR, In Vitro

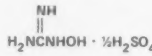
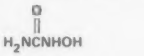
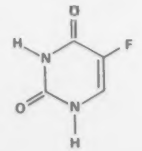
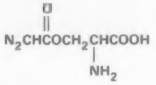
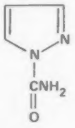
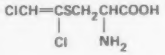
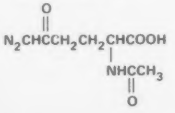
A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Amide Donors Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
49	Guanidine, hydroxy-, hemisulfate salt (NSC-44,310)	1.0	0	0	P388	+	18.7 x 9	10/10
							600.0 x 9	1/10
					L1210	+	25.0 x 9	10/10
							800.0 x 9	2/10
					Ca755	-	200.0 x 14	6/6
50	Urea, hydroxy- (NSC-32,065) HYDROXYUREA	1.0	0	0	L1210	+	200.0 x Z	6/6
		5.0	0	0			1600.0 x Z	6/6
					Ca755	+	25.0 x 11	10/10
							500.0 x 11	3/10
					LL	-	25.0 x 11	6/6
							200.0 x 11	5/6
					P388	-	31.3 x 10	6/6
							500.0 x 10	7/7
51	Cyclopentanecarboxylic acid, 1-amino- (NSC-1,026)	1.0	0	0	L1210	+	187.0 x 1	4/4
							3000.0 x 1	4/4
					L5178Y	-	22.5 x 10	6/6
							180.0 x 10	6/6
					LL	+	30.0 x 11	10/10
							120.0 x 11	7/10
52	Uracil, 5-fluoro- (NSC-19,893) FLUOROURACIL	1.0	0	0	L1210	+	75.0 x 1	8/8
							833.0 x 1	8/8
					Ca755	+	20.0 x 11	10/10
							80.0 x 11	0/10

TABLE 3. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
53	L-Serine, diazoacetate (NSC-742) AZASERINE 	1.0	0	0	Gardner 6C3HED lympho-sarcoma	+	3.13 x 10 25.0 x 10	6/6 6/6
					L1210	+	14.0 x 1 112.0 x 1	6/6 0/6
					L5178Y	+	1.0 x 6 16.0 x 6	6/6 6/6
					P388	+	3.13 x 10 25.0 x 10	6/6 6/6
54	Pyrazole-1-carboxamide (NSC-36,539) 	1.0	0	0	Ca755	+	80.0 x 11	9/10
					L1210	-	25.0 x 2 800.0 x 2	6/6 2/6
					LL	-	25.0 x 2 200.0 x 2	6/6 1/6
					P388	-	30.0 x 10 250.0 x 10	6/6 3/6
55	L-Alanine, 3-(1,2-dichloro-vinylthio)- (NSC-15,830) 	1.0 2.0 5.0	0 0 9	0 0 16	P388	+	17.0 x 9 40.0 x 9	6/6 2/6
					L1210	-	1.5 x 9 40.0 x 9	6/6 5/6
					S180	-	7.5 x 14 250.0 x 14	6/6 0/6
56	Norleucine, N-acetyl-6-diazo-5-oxo-, L- (NSC-51,097) DUAZOMYCIN A 	1.0	0	0	S180	+	0.03 x 8 0.20 x 8	6/6 6/6
					L1210	+	1.0 x 5 16.0 x 5	10/10 4/10
					L5178Y	-	0.5 x 2 2.0 x 2	6/6 6/6

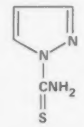
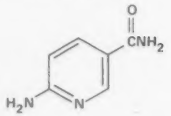
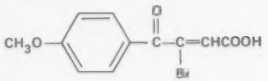
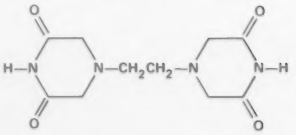
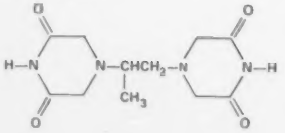
57	Pyrazole-1-carboxamide, thio- (NSC-45,409) 	1.0	0	0	Ca755	+	20.0 x 11 160.0 x 11	10/10 7/10
					L1210	-	120.0 x Z	5/6
					LL	-	100.0 x 11	6/6
					P388	-	50.0 x Z 100.0 x Z	6/6 6/6
					S180	-	60.0 x 14	5/6
58	Nicotinamide, 6-amino- (NSC-21,206) 	1.0 5.0	0 15	0 16	P1534 leukemia	+	112.0 x Z 224.0 x Z	7/7 3/7
					L1210	-	25.0 x 1 200.0 x 1	4/4 1/4
					Ca755	-	0.5 x 11 4.0 x 11	9/10 5/10
					LL	-	1.0 x 11 8.0 x 11	6/6 0/6
					S180	-	0.5 x 14 2.0 x 14	6/6 2/6
59	Acrylic acid, 3-p-anisoyl-3-bromo-, sodium salt (NSC-104,801) CYTEMBENA 	1.0	0	0	P388	+	12.5 x 9 100.0 x 9	6/6 2/6
					LL	-	12.5 x 11 160.0 x 11	8/8 0/8
					L1210	-	12.5 x 3 400.0 x 3	6/6 0/6
60	2,6-Piperazinedione, 4,4'-ethylenedi- (NSC-129,942) 	1.0 2.0	0 0	0 0	P388	+	100.0 x 3 800.0 x 3	8/8 8/8
		5.0	0	0	L1210	+	100.0 x 3 800.0 x 3	8/8 8/8
					LL	-	12.4 x 11 100.0 x 11	8/8 3/8
61	2,6-Piperazinedione, 4,4'-propylenedi-, (+)- (NSC-129,943) ICRF-159 	1.0 2.0	0 0	0 0	L1210	+	50.0 x 3 400.0 x 3	8/8 8/8
		5.0	0	0	P388	+	100.0 x 3 800.0 x 3	8/8 8/8
					LL	-	12.5 x 11 100.0 x 11	7/8 1/8

TABLE 3. (Continued)

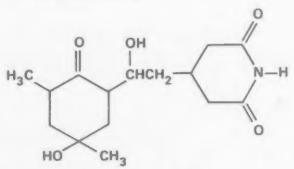
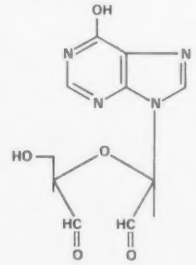
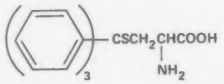
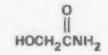
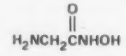
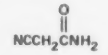
A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
62	Glutarimide, 3-[2-hydroxy-2-(5-hydroxy-3,5-dimethyl-2-oxocyclohexyl)ethyl]- (NSC-39,147) STREPTOVITACIN A 	1.0	12	15	L1210	+	3.0 x 1 65.0 x 1	10/10 0/10
					P388	+	0.1 x 10 0.8 x 10	6/6 6/6
					S180	-	0.12 x 14 0.5 x 14	5/6 1/6
63	Diglycolaldehyde, α-(hydroxymethyl)-α'-(6-hydroxy-9H-purin-9-yl)- (NSC-118,994) 	1.0	0	0	B16 melanocarcinoma	-	37.5 x 9 300.0 x 9	6/6 6/6
					L1210	+	35.0 x 1 560.0 x 1	8/8 1/8
					LL	-	37.5 x 1 300.0 x 1	8/8 0/8
					P388	+	37.5 x 10 300.0 x 10	6/6 6/6
64	L-Alanine, 3-(tritylthio)- (NSC-83,265) TRITYL-CYSTEINE 	1.0	0	0	L1210	+	17.0 x 9 140.0 x 9	8/8 3/8
					P388	+	12.5 x 9 200.0 x 9	6/6 2/6
					LL	-	12.5 x 11 200.0 x 11	6/8 0/8

TABLE 4. Compounds Lacking Both Antitumor Activity and Inhibitory Activity Versus L-Asparagine Synthetase, In Vitro

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
65	Hydrazine, dihydrochloride (NSC-2122)	1.0	0	0	S180	-	25.0 x 14	6/6
	$H_2NNH_2 \cdot 2HCl$	10.0	0	0	Walker carcino-sarcoma 256	-	20.0 x 4 400.0 x 4	6/6 0/6
66	Acetonitrile, amino-, mono sodium sulfate (1:1) (NSC-128389)	1.0	0	0	L1210	-	400.0 x 9	6/6
	$H_2NCH_2CN \cdot NaHSO_4$							
67	Sodium vanadate, hydrate (NSC-79534)	1.0	0	0	L1210	-	32.0 x 15 256.0 x 15	6/6 0/6
	$Na_3VO_4 \cdot 16H_2O$				S180	-	25.0 x 7 500.0 x 7	6/6 0/6
					Walker carcino-sarcoma 256	-	1.0 x 4 256.0 x 4	6/6 0/6
68	Acetic acid, (aminooxy)-, hemihydro-chloride (NSC-40358)	1.0	0	0	Ca755	-	13.5 x 11 27.0 x 11	10/10 0/10
	$H_2NOCH_2COOH \cdot \frac{1}{2}HCl$				L1210	-	5.0 x 2 27.0 x 2	6/6 6/6
					S180	-	7.45 x 14	5/6
69	Allophanic acid, hydrazide (NSC-17298) BIURET, 1-AMINO-	1.0	0	0	Ca755	-	115.0 x 11	9/10
					L1210	-	125.0 x 2	6/6
	$H_2NCNHCNHNH_2$ (O, O)				S180	-	62.5 x 14 250.0 x 14	6/6 0/6
70	Guanidine, sulfate (NSC-7296)	1.0	0	0	Ca755	-	225.0 x 11 450.0 x 11	10/10 0/10
	$H_2NCNH_2 \cdot H_2SO_4$ (NH)				L1210	-	125.0 x 2 500.0 x 2	6/6 2/6
					S180	-	250.0 x 14	6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
71	Glycine (NSC-25936)	1.0	0	0	L1210	-	100.0 x 1 325.0 x 9	6/6 6/6
					L5178Y	-	187.0 x 20 1500.0 x 20	10/10 10/10
					P388	-	100.0 x 3 400.0 x 3	6/6 6/6
					S180	-	125.0 x 14	4/6
					Gardner 6C3HED lympho-sarcoma	-	187.0 x 20 1500.0 x 20	6/6 6/6
72	Oxamic acid (NSC-47001)	1.0	0	0	Ca755	-	300.0 x 11	10/10
					L1210	-	300.0 x Z	6/6
					S180	-	187.0 x 14	5/6
73	Glycolamide (NSC-97323) 	1.0	0	0	L1210	-	500.0 x Z	6/6
					LL	-	500.0 x 11	6/6
					Dunning leukemia	-	500.0 x 5	6/6
74	Acetohydroxamic acid, 2-amino- (NSC-3841) 	1.0	0	0	L1210	-	90.0 x 15 1000.0 x 15	6/6 0/6
					Walker carcino-sarcoma 256	-	148.0 x 3 500.0 x 3	6/6 5/6
75	Acetamide, 2-cyano- (NSC-6285) 	1.0	0	0	Ca755	-	100.0 x 11 450.0 x 11	10/10 5/10
					L1210	-	125.0 x Z 500.0 x Z	6/6 1/6
					S180	-	250.0 x 14	6/6

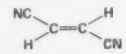
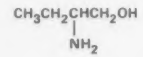
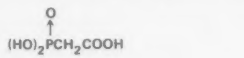
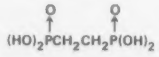
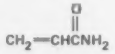
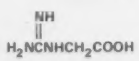
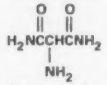
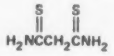
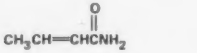
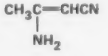
76	Fumaronitrile (NSC-17555) 	1.0	0	0	Ca755	-	15.0 x 11 30.0 x 11	9/10 5/10
					L1210	-	0.15 x 9 450.0 x Z	6/6 0/6
					P388	-	0.15 x 9 0.6 x 9	6/6 6/6
					S180	-	17.5 x 14 250.0 x 14	9/12 0/6
77	1-Butanol, 2-amino- (NSC-1068) 	1.0	0	0	Ca755	-	125.0 x 11	6/6
					L1210	-	100.0 x 1 400.0 x 1	4/4 2/4
					S180	-	125.0 x 14 250.0 x 14	5/6 6/10
78	Acetic acid, fluoro-, sodium salt (NSC-77690)	1.0	0	0	L1210	-	4.0 X Z 500.0 x Z	5/6 0/6
					Walker carcino-sarcoma 256	-	0.2 x 4 4.0 x 4	4/6 0/6
79	Acetic acid, phosphono- (NSC-18205) 	1.0	0	0	Ca755	-	112.0 x 11	10/10
					L1210	-	62.5 x Z 125.0 x Z	6/6 6/6
					S180	-	62.5 x 14 250.0 x 14	6/6 3/6
80	Phosphonic acid, ethylenedi- (NSC-40837) 	1.0	0	0	Ca755	-	112.0 x 11	10/10
		5.0	0	0	L1210	-	112.0 x Z	6/6
					S180	-	62.5 x 6	6/6
81	Acrylamide (NSC-7785) 	1.0 5.0	0 15	0 0	Ca755	-	10.0 x 11 80.0 x 11	10/10 0/10
					L1210	-	35.0 x Z	6/6
					S180	-	22.5 x 14 250.0 x 14	6/6 0/6
82	Glycine, N-amidino- (NSC-26360) GLYCOCYAMINE 	1.0	0	0	Ca755	-	450.0 x 11	9/10
					L1210	-	450.0 x Z	6/6
					S180	-	250.0 x 14	7/7

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
83	Malonamide, 2-amino- (NSC-75208) 	1.0	0	0	L1210	-	50.0 x Z 100.0 x Z	6/6 6/6
					S180	-	125.0 x 7	6/6
84	Malonamide, dithio- (NSC-55978) 	1.0	13	25	Ca755	-	450.0 x 11	9/10
					L1210	-	450.0 x Z	6/6
					S180	-	250.0 x 14	6/6
85	Acetylenedicarboxylic acid (NSC-1903) $HOOCC{\equiv}CCOOH$	1.0	0	0	Ca755	-	18.0 x 11 30.0 x 11	6/6 3/6
					L1210	-	3.0 x Z 100.0 x Z	5/6 5/5
					S180	-	7.5 x 14 250.0 x 14	6/6 0/10
					Walker carcino-sarcoma 256	-	50.0 x 7 400.0 x 7	6/6 6/6
86	Crotonic acid (NSC-8751) $CH_3CH{=}CHCOOH$	1.0	0	0	Ca755	-	95.0 x 11	9/10
					L1210	-	105.0 x Z	6/6
					S180	-	52.5 x 14 250.0 x 14	6/6 0/6
87	Crotonamide (NSC-11802) 	1.0	0	0	Ca755	-	400.0 x 11	8/10
					L1210	-	500.0 x Z	6/6
					S180	-	250.0 x 14	5/6
88	Crotononitrile, 3-amino- (NSC-102759) 	1.0	0	0	L1210	-	40.0 x 15 50.0 x 1 400.0 x 1	6/6 3/4 0/4
					Walker carcino-sarcoma 256	-	40.0 x 4	6/6

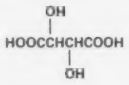
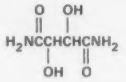
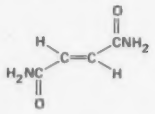
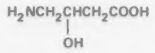
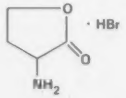
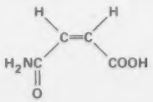
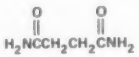
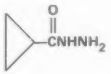
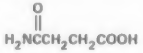
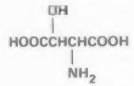
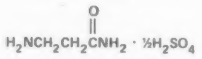
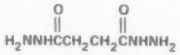
89	L-Tartaric acid (NSC-62778) 	1.0	0	0	Ca755 L1210 S180	- - -	110.0 x 11 110.0 x Z 62.5 x 14 250.0 x 14	10/10 6/6 6/6 1/6
90	Tartaramide (NSC-38709) 	1.0	0	0	Ca755 L1210 S180	- - -	450.0 x 11 450.0 x Z 250.0 x 14	9/10 6/6 6/6
91	Fumaramide (NSC-16608) 	1.0	0	0	Ca755 L1210	- -	29.0 x 11 230.0 x 11 450.0 x Z	9/10 0/6 6/6
92	Diglycolic acid (NSC-8651) $HOOCCH_2OCH_2COOH$	1.0	0	0	Ca755 L1210 S180	- - -	100.0 x 11 400.0 x 11 250.0 x Z 500.0 x Z 250.0 x 14	10/10 1/10 6/6 2/6 5/6
93	Butyric acid, 4-amino-3-hydroxy- (NSC-40244) 	1.0	0	0	Ca755 L1210 S180	- - -	450.0 x 11 450.0 x Z 250.0 x 14	10/10 6/6 6/6
94	Butyric acid, 2-amino-4-hydroxy-, γ-lactone, hydrobromide (NSC-41120) 	1.0	0	0	Ca755 L1210 S180	- - -	400.0 x 11 400.0 x Z 250.0 x 14	10/10 6/6 5/6
95	Maleamic acid (NSC-8155) 	1.0	0	0	Ca755 L1210 S180	- - -	225.0 x 11 450.0 x 11 500.0 x Z 250.0 x 14	10/10 2/10 5/6 6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Amide Donors Ammonia	Antitumor Activity Tumor System	Antitumor Activity Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
96	Succinamide (NSC-8157) 	10.0	0	0	Ca755	-	450.0 x 11	10/10
					L1210	-	500.0 x 2	7/7
					S180	-	250.0 x 14	6/6
97	Cyclopropanecarboxylic acid, hydrazide (NSC-70850) 	1.0	0	0	Ca755	-	50.0 x 11 200.0 x 1	10/10 0/10
					L1210	-	40.0 x 2 200.0 x 2	6/6 0/6
					S180	-	50.0 x 7 250.0 x 7	6/6 0/6
98	Succinamic acid (NSC-78416) 	1.0	0	0	L1210	-	400.0 x 2	6/6
					S180	-	500.0 x 7	4/6
					Friend virus leukemia (solid)	-	400.0 x 11	9/10
99	Aspartic acid, 3-hydroxy-, DL-threo- (NSC-119123) 	1.0	0	0	L1210	-	125.0 x 9 400.0 x 1	6/6 6/6
100	β-Alaninamide, sulfate (2:1) (NSC-136519) 	1.0	0	0	L1210	-	400.0 x 9	6/6
					L5178Y	-	50.0 x 5 400.0 x 5	10/10 10/10
					P388	-	100.0 x 9 400.0 x 9	6/6 6/6
101	Succinic acid, dihydrazide (NSC-25180) 	1.0	0	0	Ca755	-	100.0 x 11	11/12
					L1210	-	50.0 x 2 100.0 x 2	6/6 6/6
					S180	-	62.5 x 14 250.0 x 14	5/6 0/6

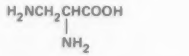
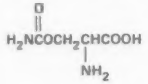
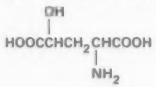
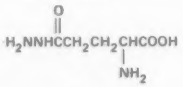
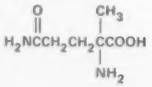
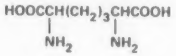
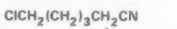
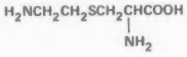
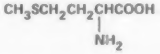
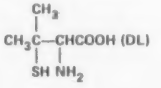
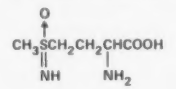
102	Acrylic acid, cis-3-amidino- (NSC-93048)	1.0	0	0	L1210		24.0 x Z	6/6
					LL	-	24.0 x 11	6/6
					P388	-	6.0 x 10 50.0 x 10	6/6 1/6
					S180	-	7.0 x 7 60.0 x 7	5/6 0/6
103	Propionic acid, 2,3-diamino-, (-)- (NSC-115849) 	1.0	0	0	L1210	-	100.0 x 1 400.0 x 9	4/4 6/6
104	Serine, carbamate (ester), L- (NSC-128373) 	1.0	0	0	L1210	-	75.0 x 1 300.0 x 1	6/6 6/6
					L5178Y	-	50.0 x 5 400.0 x 5	10/10 10/10
105	DL-Glutamic acid, 4-hydroxy- 	1.0	0	0	Ca755	-	400.0 x 11	9/10
106	Glutamic acid, 5-hydrazide, L- (NSC-7786) 	0.5 1.0	0 0	0 0	Ca755	-	10.2 x 11 104.0 x 11	10/10 8/9
		10.0	0	0	L1210	-	10.7 x Z 215.0 x Z	10/10 7/10
					P388	-	50.0 x 9 200.0 x 9	6/6 5/6
					S180	-	5.75 x 14 46.0 x 14	10/10 9/10
					Walker carcino-sarcoma 256	-	200.0 x 4 800.0 x 4	6/6 0/6
107	Glutamine, 2-methyl- (NSC-42197) 	1.0	0	0	L1210	-	450.0 x Z	6/6
					S180	-	250.0 x 14	6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
108	Heptanedioic acid,2,6-diamino (NSC-45225) 	10.0	0	0	Ca755	-	180.0 x 11 600.0 x 11	10/10 10/10
					L1210	-	180.0 x 2	6/6
					S180	-	100.0 x 14	6/6
109	Hexanenitrile, 6-chloro- (NSC-59713) 	1.0	0	0	Ca755	-	150.0 x 11 300.0 x 11	9/10 0/10
					L1210	-	300.0 x 2	5/6
					S180	-	187.0 x 14	5/6
110	Alanine, 3-[(2-aminoethyl)-thio]- (NSC-115857) 	1.0	0	0	L1210	-	200.0 x 9 400.0 x 9	6/6 6/6
111	Methionine, L(-)- (NSC-22946) 	1.0	0	0	Ca755	-	400.0 x 11	10/10
					L1210	-	100.0 x 1 400.0 x 1	6/6 6/6
					S180	-	250.0 x 14	5/6
112	Valine, 3-mercapto-, DL- (NSC-44656) 	1.0	0	0	Ca755	-	31.0 x 11 250.0 x 11	10/10 0/10
					L1210	-	20.0 x 2	6/6
					S180	-	250.0 x 14	0/6
113	Sulfoximine, S-(L-3-amino-3-carboxypropyl)-S-methyl L-METHIONINE-DL-SULFOXIMINE 	1.0	0	0	L1210	-	25.0 x 3 100.0 x 3	6/6 2/6
					P388	-	6.25 x 9 400.0 x 9	6/6 6/6
					Walker carcino-sarcoma 256	-	12.0 x 4	6/6

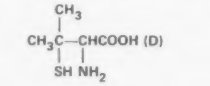
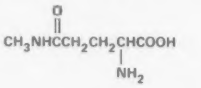
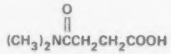
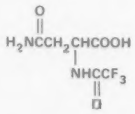
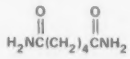
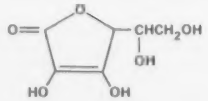
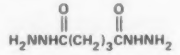
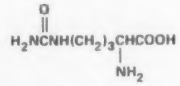
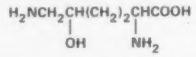
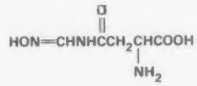
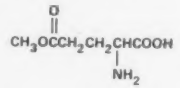
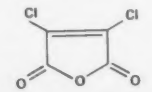
114	Valine, 3-mercapto-, D- (NSC-81549) 	1.0	0	0	L1210	-	100.0 x 9 400.0 x Z	6/6 6/6
					P388	-	100.0 x 3 400.0 x 3	6/6 6/6
					S180	-	125.0 x 7 1500.0 x 7	7/7 7/7
115	Acrylamide, 3-glyoxylamido-, oxime, trans- (NSC-85680) ZEDALAN	1.0	0	0	L1210	-	12.5 x Z 400.0 x 15	6/6 6/6
					L5178Y	-	50.0 x 8 200.0 x 8	6/6 6/6
116	Glutamine, N-methyl- (NSC-21552) 	1.0	0	0	Ca755	-	100.0 x 11	9/10
					L1210	-	62.5 x Z 125.0 x Z	6/6 6/6
					S180	-	62.5 x 14 250.0 x 14	5/6 3/6
117	Succinamic acid, N,N-dimethyl- (NSC-46778) 	1.0	0	0	Ca755	-	112.0 x 11	9/10
					L1210	-	112.0 x Z	6/6
					S180	-	62.5 x 14 250.0 x 14	6/6 3/6
118	Asparagine, N2-(trifluoroacetyl)-, DL- (NSC-522627+) 	1.0	0	0	L1210	-	75.0 x 1 400.0 x 1	6/6 3/6
119	Adipamide (NSC-7623) 	1.0	0	0	Ca755	-	350.0 x 11	10/10
					L1210	-	500.0 x Z	6/6
					S180	-	250.0 x 14	5/6
120	Ascorbic acid (NSC-33832) 	1.0	0	0	Ca755	-	450.0 x 11	8/10
					L1210	-	400.0 x 9 1600.0 x 9	6/6 0/6
					P388	-	0.13 x 9 400.0 x 9	6/6 3/6
					S180	-	250.0 x 14	6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
121	Glutaric acid, dihydrazide 	1.0	0	0	L1210	-	400.0 x 1	6/6
122	Valeric acid, L-2-amino-5-ureido- (NSC-27425) L-CITRULLINE 	1.0	0	0	L1210	-	450.0 x Z	6/6
					P388	-	50.0 x 10 400.0 x 10	6/6 4/6
					S180	-	250.0 x 14	6/6
					Walker carcino-sarcoma 256	-	25.0 x 4 200.0 x 4	6/6 6/6
123	Lysine, 5-hydroxy-, monohydro-chloride, DL-allo-(+)- (NSC-132938) 	1.0	0	0	L1210	-	400.0 x 9	5/6
124	Succinamic Acid, DL-2-amino-N-formyl-, oxime (NSC-100879) 	1.0	0	0	L1210	-	50.0 x 1 400.0 x 1	4/4 4/4
125	L-Glutamic acid, 5-methyl ester (NSC-12959) 	1.0	0	0	Ca755	-	175.0 x 11 350.0 x 11	10/11 6/10
					L1210	-	500.0 x Z	6/6
					S180	-	250.0 x 14	4/6
126	Maleic anhydride, dichloro- (NSC-60524) 	1.0	0	0	Ca755	-	100.0 x 11	7/10
					L1210	-	100.0 x Z	6/6
					S180	-	62.5 x 14 250.0 x 14	5/6 0/6

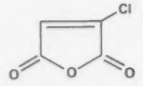
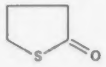
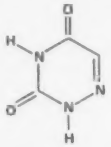
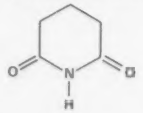
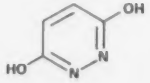
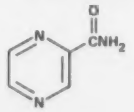
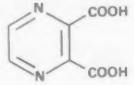
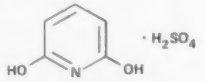
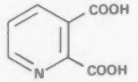
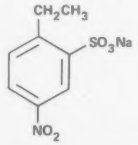
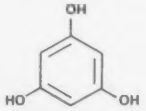
127	Maleic anhydride, chloro- (NSC-4975) 	1.0	0	0	Ca755	-	260.0 x 6	0/10
128	Butyric acid, 4-mercapto-, γ-(thio lactone) (NSC-54087) 	1.0	0	0	Ca755	-	40.0 x 11	7/10
					L1210	-	40.0 x Z	6/6
					S180	-	25.0 x 14 250.0 x 14	5/6 0/6
129	as-Triazine-3,5(2H,4H)-dione (NSC-3425) 6-AZAURACIL 	1.0	0	0	Ca755	-	100.0 x 11 1500.0 x 11	10/10 5/10
					Gardner 6C3HED lympho-sarcoma	-	62.5 x 10 500.0 x 10	6/6 6/6
					L1210	-	62.5 x 10 1000.0 x Z	6/6 6/6
					L5178Y	-	62.5 x 10 500.0 x 10	6/6 6/6
130	Glutarimide (NSC-58190) 	1.0	0	0	Ca755	-	50.0 x 11 100.0 x 11	10/10 5/10
					L1210	-	100.0 x Z	5/6
					S180	-	62.5 x 14	5/6
131	3,6-Pyridazinediol (NSC-8823) 	1.0	0	0	Ca755	-	450.0 x 11	9/10
					L1210	-	100.0 x 9 400.0 x 9	6/6 5/6
					S180	-	250.0 x 14	6/6
132	Pyrazinamide (NSC-14911) PYRAZINECARBOXAMIDE 	1.0	0	0	Ca755	-	50.0 x 11	10/10
					L1210	-	60.0 x Z	6/6
					S180	-	30.0 x 14 250.0 x 14	5/6 3/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Amide Donors Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
133	2,3-pyrazinedicarboxylic acid (NSC-1908) 	1.0	0	0	Ca755	-	53.0 x 11 125.0 x 11	6/6 4/6
					L1210	-	75.0 x Z 150.0 x Z	6/6 2/6
					S180	-	75.0 x 14 250.0 x 14	10/10 2/10
					Walker carcino sarcoma 256	-	100.0 x 4 400.0 x 4	6/6 0/6
134	2,6-Pyridinediol, sulfate (NSC-71877) 	1.0	0	0	L1210	-	80.0 x Z	6/6
					LL	-	80.0 x 11	6/6
					S180	-	100.0 x 7 500.0 x 7	6/6 0/6
135	2,3-Pyridinedicarboxylic acid (NSC-13127) QUINOLINIC ACID 	1.0 10.0	0 0	0 0	Ca755	-	110.0 x 11 450.0 x 11	10/10 0/10
					L1210	-	500.0 x Z	5/6
					S180	-	250.0 x 14	6/6
136	Benzenesulfonic acid, 2-ethyl-5-nitro-, sodium salt 	1.0	0	0	L1210	-	400.0 x 9	6/6
137	Phloroglucinol (NSC-1572) 	1.0	0	0	Ca755	-	350.0 x 11	9/10
					L1210	-	500.0 x Z	7/7
					S180	-	250.0 x 14	8/10
					Walker carcino-sarcoma 256	-	400.0 x 4	7/7

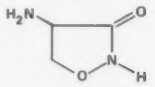
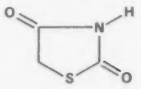
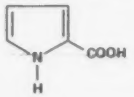
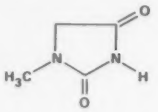
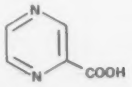
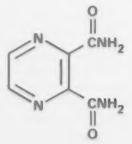
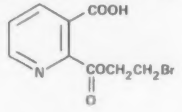
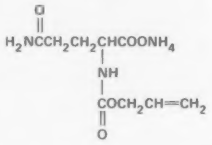
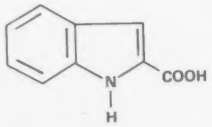
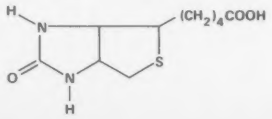
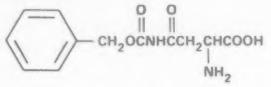
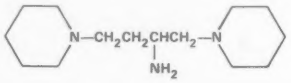
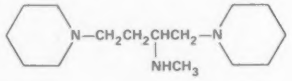
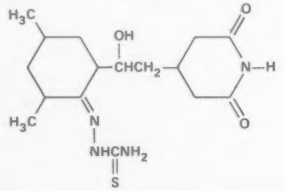
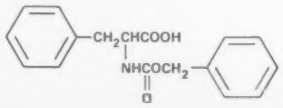
138	3-Isoxazolidinone, 4-amino- (NSC-76029) CYCLOSERINE 	1.0	0	0	L1210	-	400.0 x Z	6/6
					S180	-	500.0 x 7	6/6
					Friend virus leukemia (solid)	-	400.0 x 11	10/10
139	2,4-Thiazolidinedione (NSC-6745) 	1.0	0	0	Ca755	-	87.5 x 11	10/10
		5.0	0	0	L1210	-	100.0 x Z	6/6
					S180	-	62.5 x 14 250.0 x 14	6/6 0/6
140	Pyrrole-2-carboxylic acid (NSC-48130) 	1.0	0	0	Ca755	-	350.0 x 11	7/10
					L1210	-	350.0 x Z	5/6
					S180	-	250.0 x 14	4/6
141	Hydantoin, 1-methyl- (NSC-80560) 	1.0	0	0	Ca755	-	250.0 x 11 2000.0 x 11	10/10 6/10
					L1210	-	250.0 x 7 1000.0 x 7	7/7 7/7
					LL	-	250.0 x 11 2000.0 x 11	7/7 7/7
142	Pyrazinecarboxylic acid (NSC-27192) 	1.0	0	0	Ca755	-	113.0 x 11 225.0 x 11	8/10 0/10
					L1210	-	113.0 x Z 225.0 x Z	6/6 6/6
					S180	-	250.0 x 14	3/6
143	2,3-Pyrazinedicarboxamide (NSC-93820) 	1.0	0	0	L1210	-	200.0 x 5 400.0 x Z	6/6 6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
144	2,3-Pyridinedicarboxylic acid, 2-(2-bromoethyl) ester (NSC-49916) 	1.0	0	0	Ca755	-	112.0 x 11 225.0 x 11	10/10 2/12
					L1210	-	225.0 x Z	6/6
					S180	-	125.0 x 14 250.0 x 14	6/6 2/6
145	Glutamine, N2-carboxy-, N-allyl ester, ammonium salt (NSC-12962) 	1.0	0	0	Ca755	-	350.0 x 11	10/10
					L1210	-	500.0 x Z	6/6
					S180	-	250.0 x 14	6/6
146	Indole-2-carboxylic acid (NSC-16598) 	1.0	0	0	L1210	-	500.0 x Z	5/6
					S180	-	250.0 x 14	6/6
147	(+)-Biotin Thieno[3,4-d]imidazoline-4-valeric acid, (+)-cis-tetrahydro-2-oxo- (NSC-63865) 	1.0	0	0	Ca755	-	116.0 x 11 231.0 x 11	10/10 5/10
					L1210	-	231.0 x Z	6/6
					S180	-	330.0 x 7	4/6

148	Pyrazole-3-carboxamide, 4-hydroxy-5-β-D-ribofuranosyl- (NSC-143095) PYRAZOMYCIN	1.0	0	0	L1210	-	0.25 x 3 20.0 x 3	6/6 6/6
					P388	-	0.25 x 9 80.0 x 9	6/6 0/6
149	Glutarimide, 3-[2-hydroxy-2-(5-hydroxy-3,5-dimethyl-2-oxocyclohexyl)ethyl], acetate (ester) (NSC-32743)	1.0	0	0	L1210	-	0.06 x 9 2.0 x 9	6/6 1/6
					P388	-	0.13 x 10 0.25 x 10	6/6 6/6
150	Adenosine cyclic 3',5'-phosphate (NSC-94017)	1.0	0	0	L1210	-	50.0 x 9 400.0 x 9	5/6 2/6
151	Riboflavin (NSC-33298)	1.0	0	0	Ca755	-	225.0 x 11 450.0 x 11	8/10 5/10
					L1210	-	25.0 x 9 400.0 x 9	6/6 0/6
					P388	-	100.0 x 10 800.0 x 10	4/6 0/6
					S180	-	500.0 x 7	6/6

TABLE 4. (Continued)

A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%) Concentration mM	Amide Donors L-Glutamine	Ammonia	Antitumor Activity Tumor System	Activity	Dose and Regimen (mg/kg x days)	Survivors/ No. Tested
152	Asparagine, N-carboxy-, N-benzyl ester, L- (NSC-136953) 	1.0	0	0	L1210	-	400.0 x 9	6/6
153	Piperidine, 1,1'-(2-aminotetramethylene)-bis-, trihydrochloride, monohydrate (NSC-138352) 	1.0	0	0	L1210	-	400.0 x 1	6/6
154	Piperidine, 1,1'-[2-(methlamino)tetra-methylene]di-, trihydrochloride (NSC-138353) 	1.0	0	0	L1210	-	400.0 x 9	4/6
155	Glutarimide, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-, thiosemi-carbazone (NSC-17254) ACTIDIONE THIOSEMICARBAZONE 	1.0	0	0	Ca755	-	450.0 x 11	7/10
					L1210	-	25.0 x 9	6/6
							400.0 x 9	6/6
					S180	-	250.0 x 14	5/6
156	L-Alanine, N-carboxy-3-phenyl-, N-benzyl ester (NSC-402059) 	1.0	0	0	L1210	-	200.0 x 2	6/6
		5.0	0	13	LL	-	50.0 x 11	6/6
							200.0 x 11	1/6
					S180	-	250.0 x 7	6/6
							500.0 x 7	2/6

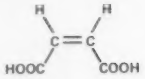
156a	Methylamine hydrochloride (NSC-89685)	1.0	0	0	L1210	-	125.0 x Z 250.0 x Z	6/6 6/6
	$CH_3NH_2 \cdot HCl$				LL	-	125.0 x 11 250.0 x 11	6/6 3/6
					S180	-	250.0 x 7 500.0 x 7	6/6 3/6
156b	Malonic acid (NSC-8124)	1.0	0	0	Ca755	-	70.0 x 11	9/10
	$HOOCCH_2COOH$				L1210	-	100.0 x Z	6/6
					S180	-	51.5 x 14 250.0 x 14	6/6 0/6
156c	Maleic acid (NSC-25940)	1.0 10.0	0 0	- -	Ca755	-	45.0 x 11 90.0 x 11	10/10 6/10
					L1210	-	50.0 x 3 400.0 x 3	6/6 0/6
					P388	-	25.0 x 9 100.0 x 9	6/6 6/6
156d	Malic acid	1.0 10.0	0 0	- -	Ca755	-	135.0 x 11 270.0 x 11	9/10 0/10
	$HOOCCH_2CHCOOH$ $\quad\quad\quad

TABLE 5. Compounds Inhibiting L-Asparagine Synthetase from L5178Y/AR, In Vitro but for which Antitumor Data are Unavailable

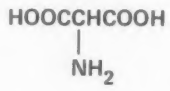
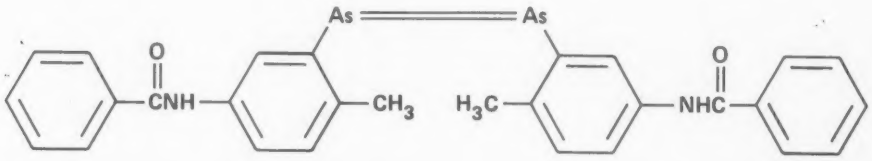
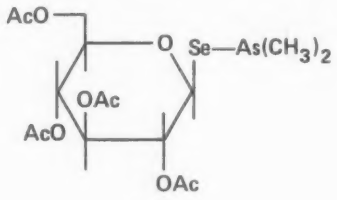
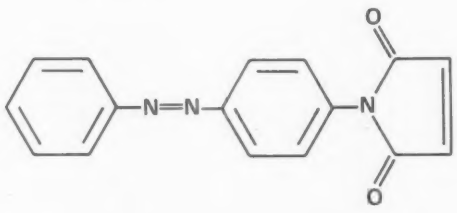
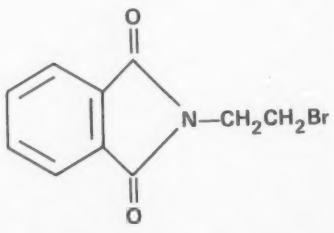
A Numbers	Compound	Inhibition of L-Asparagine Synthetase (%)		
		Concentration mM	Amide Donors	
			L-Glutamine	Ammonia
157	Aminomalonic acid	1.0	70	60
				
158	*p*-Benzotoluidine, 3',3'''-arsenobis- (NSC-92920)	1.0	50	84
				
159	β-D-Glucopyranose, 1-seleno-2,3,4,6-tetraacetate, 1-(dimethylarsenite) (NSC-163665)	1.0	53	35
				
160	*p*-Phenylazomaleinanil	1.0	55	55
				
161	*N*-(2-Bromoethyl)-phthalimide	1.0	50	65
				

TABLE 5. (Continued)

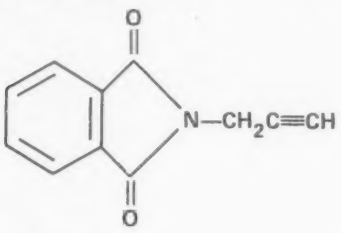
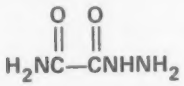
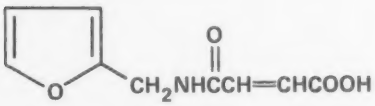
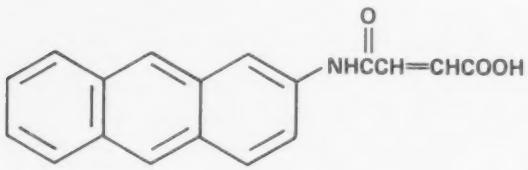
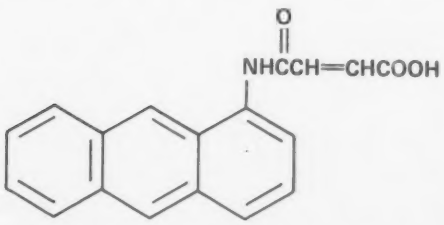
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
162	*N*-Propargylphthalimide 	1.0	55	66
163	Oxamic hydrazide 	1.0	20	21
164	*N*-Furfuryl-maleamic acid 	1.0	70	80
165	2-Aminoanthracene maleamic acid 	1.0	58	42
166	1-Aminoanthracene maleamic acid 	1.0	44	45

TABLE 5. (Continued)

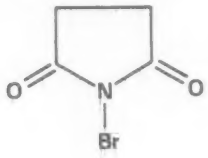
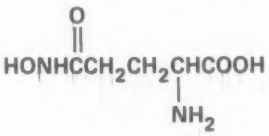
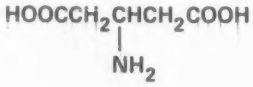
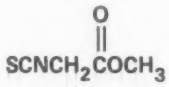
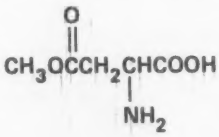
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors: L-Glutamine	Amide Donors: Ammonia
167	*N*-Bromosuccinimide 	0.1	31	30
		1.0	91	100
168	*p*-Chloromercuriphenyl sulfonic acid	0.01	0	0
		0.1	33	67
		0.5	100	100
		1.0	100	100
169	L-Glutamic acid γ-monohydroxamate 	1.0	0	18
		10.0	71	67
170	β-Glutamic acid (β-aminoglutaric acid) 	1.0	17	41
		10.0	77	90
171	Acetic acid, isothiocyanatemethyl ester (NSC-218,418) 	1.0	21	15
172	DL-Aspartic acid, 4-methyl ester 	1.0	25	25

TABLE 5. (Continued)

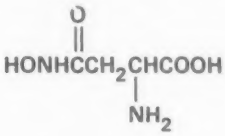
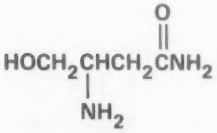
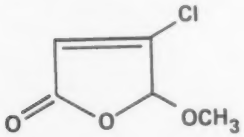
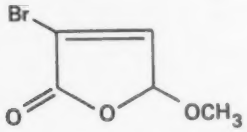
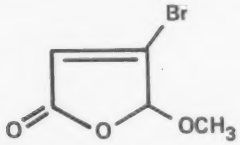
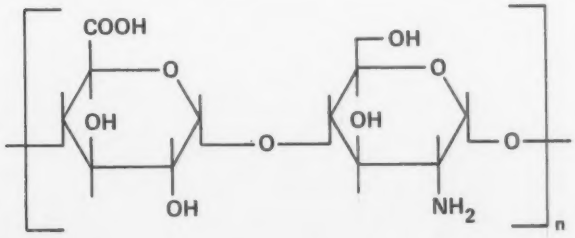
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
173	Asparagine, *N*-hydroxy-, DL- (NSC-528,507)	1.0	0	0
		10.0	77	62
				
174	Butyramide, 3-amino-4-hydroxy-, monohydrochloride, L- (NSC-143,119)	1.0	32	16
		10.0	33	40
				
175	2(5*H*)-Furanone, 4-chloro-5-methoxy-	1.0	44	67
				
176	2(5*H*)-Furanone, 3-bromo-5-methoxy-	1.0	23	42
				
176a	2(5*H*)-Furanone, 4-bromo-5-methoxy-	1.0	47	93
				
177	Heparin	200 units/ml	70	72
				

TABLE 5. (Continued)

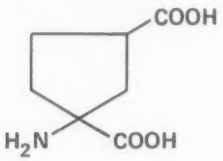
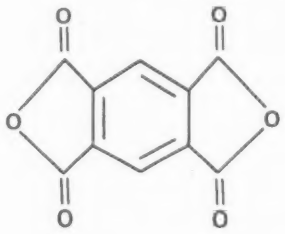
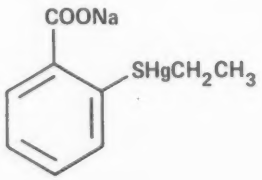
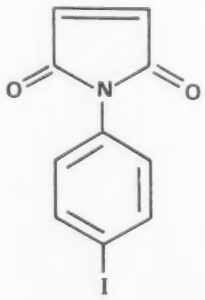
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
178	1,3-Cyclopentanedicarboxylic acid, 1-amino-, monohydrate stereoisomers (NSC-27,386)	1.0	0	29
		2.0	36	30
				
179	1*H*,3*H*-Benzo[1,2-*c*:4,5-*c'*]difuran-1,3,5,7-tetrone (NSC-4,798)	1.0	19	24
				
180	Thiomersal	1.0	97	96
				
181	*N*-4-Iodophenylmaleimide	1.0	7	27
				

TABLE 5. (Continued)

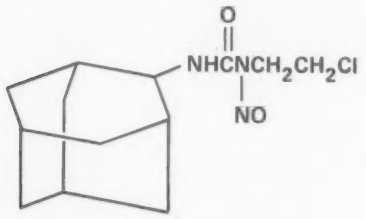
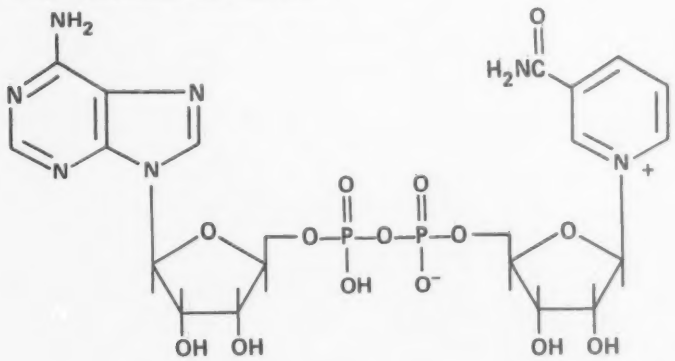
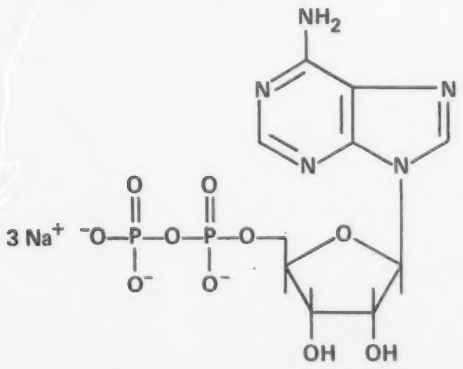
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
182	Urea, 3-(2-adamantyl)-1-(2-chloroethyl)-1-nitroso (NSC-129,968)	1.0	16	31
				
183	Zinc sulfate	0.01	0	13
		0.1	58	76
	$Zn(SO_4)_2$	1.0	100	100
184	β-Diphosphopyridine nucleotide (NAD)	1.0	40	36
				
185	Adenosine-5'-diphosphate (ADP)	1.0	15	20
				

TABLE 5. (Continued)

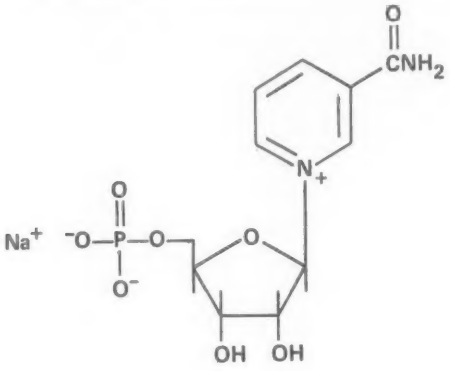
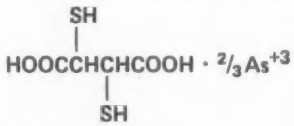
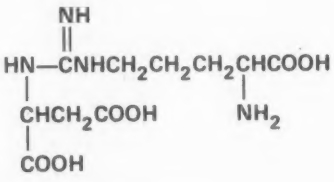
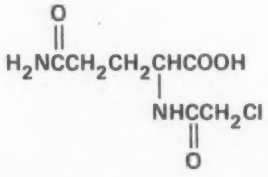
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors: L-Glutamine	Amide Donors: Ammonia
186	Nicotinamide mononucleotide (NMN)	1.0	12	14
				
187	Calcium chloride	0.1	14	17
	$CaCl_2$	1.0	75	75
188	Cobaltous chloride	0.1	0	0
	$CoCl_2 \cdot 6H_2O$	1.0	19	44
189	2,3-Dimercaptosuccinic acid, *S,S*-arsenic salt (NSC-16,866)	1.0	34	0
				
190	Argininosuccinic acid	1.0	0	21
				
191	Chloroacetyl-L-glutamine	1.0	0	0
		10.0	0	24

TABLE 5. (Continued)

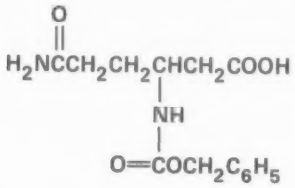
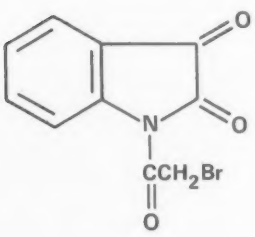
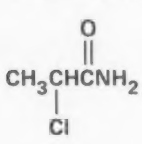
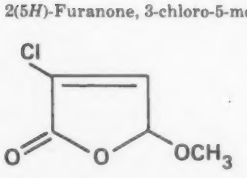
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
192	Carbobenzoxy-L-β-aminoadipamic acid	1.0	0	0
		10.0	0	24
				
193	1*H*-Indole-2,3-dione, 1-(bromoacetyl)-	1.0	0	22
				
194	DL-2-Chloropropionamide (NSC-401,803)	1.0	0	28
				
195	2(5*H*)-Furanone, 3-chloro-5-methoxy-	1.0	0	43
				

TABLE 5. (Continued)

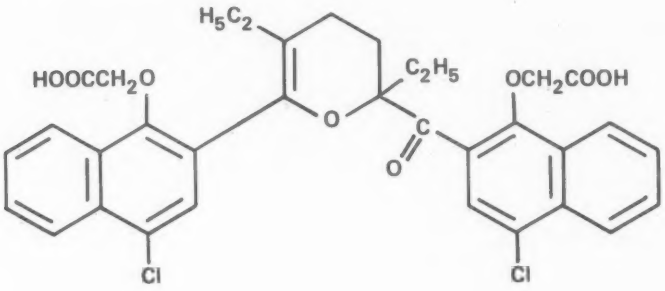
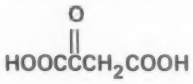
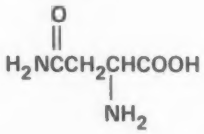
A Numbers	Compound	Concentration mM	Inhibition of L-Asparagine Synthetase (%) Amide Donors L-Glutamine	Ammonia
195a	2-(1-Carboxymethoxy-4-chloro-2-napththyl)-3,6-diethyl-6-(1-carboxymethoxy-4-chloro-2-naphthoyl)-5,6-dihydro-[4*H*]-pyran (NSC-83,143)	1.0	100	100
				
195b	Oxaloacetic acid (NSC-77688)	1.0	60	Not Tested
		10.0	90	Not Tested
				
195c	Sodium pyrophosphate	1.0	5	0
		5.0	10	19
	$Na_4P_2O_7 \cdot IOH_2O$	10.0	28	41
		20.0	98	98
195d	L-Asparagine	1.0	45	0
		10.0	90	0
		50.0	100	0

TABLE 6. Compounds Exhibiting No Inhibition of L-Asparagine Synthetase and for which Antitumor Data were Unavailable

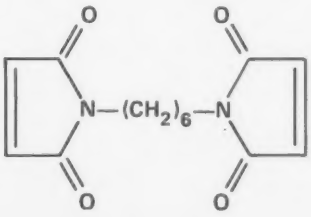
A Numbers	Compound	Concentrations Tested (mM)
196	Succinaldoxime (NSC-5567)	1.0
	$HON{=}CHCH_2CH_2CH{=}NOH$	
197	Glutaramic acid	1.0 10.0
	$H_2NC(=O)CH_2CH_2CH_2COOH$	
198	*N*-Carbamyl-L-glutamic acid	1.0
	$HOOCCH_2CH_2CH(NHC(=O)NH_2)COOH$	
199	(Dimethylaminomethylene) malononitrile	1.0
	$(CH_3)_2NCH{=}C(CN)_2$	
200	*N*-Allylmaleamic acid	1.0
	$CH_2{=}CHCH_2NHC(=O)CH{=}CHCOOH$	
201	*N,N'*-(1,4-Cyclohexylenedimethylene)-dimaleamic acid	1.0
202	*N*-Carbamylmaleamic acid	1.0
	$H_2NC(=O)NHC(=O)CH{=}CHCOOH$	
203	*N,N'*-Hexamethylenedimaleimide	1.0
		

TABLE 6. (Continued)

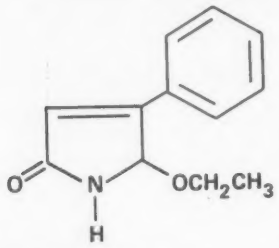
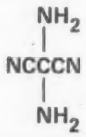
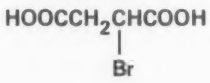
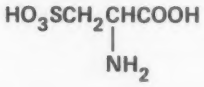
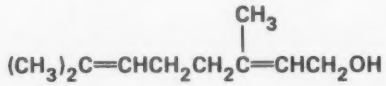
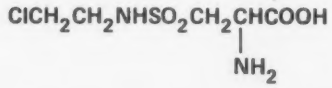
A Numbers	Compound	Concentrations Tested (mM)
204	2-Ethoxy-3-phenylmaleimide	1.0
		
205	Diaminomalononitrile	1.0
		
206	Carboxymethoxylamine hemihydrochloride	1.0
	$H_2NOCH_2COOH \cdot \frac{1}{2}HCl$	
207	2-Bromosuccinic acid	1.0
		
208	Taurine	1.0
	$H_2NCH_2CH_2SO_3H$	
209	DL-Cysteic acid	1.0
		
210	Geraniol (NSC-9279)	1.0
		
211	Molybdic anhydride	1.0
	MoO_3	
212	Alanine, 3-[(2-chloroethyl) sulfamoyl]-L- (NSC-158203)	1.0
		

TABLE 6. (Continued)

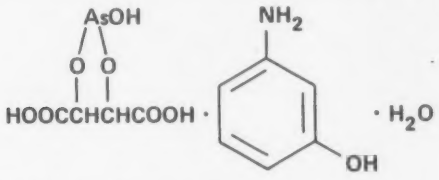
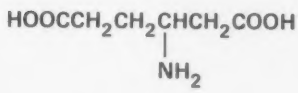
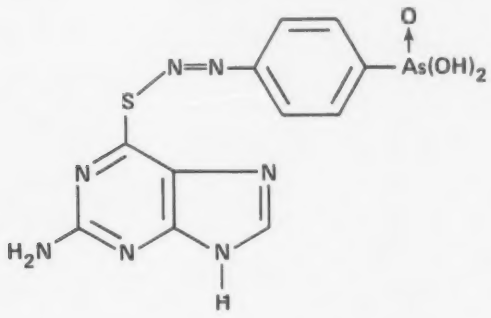
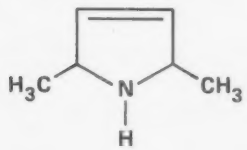
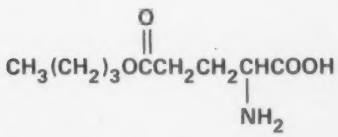
A Numbers	Compound	Concentrations Tested (mM)
213	Arsenic tartrate, salt with *m*-aminophenol, hydrate (NSC-31661)	1.0
		
214	DL-β-Aminoadipic acid	1.0 10.0
		
215	Benzenearsonic acid, *p*-([2-(aminopurine-6-yl)thio]azo)- (NSC-79193)	1.0
		
216	2,5-Dimethyl-3-pyrroline	1.0
		
217	Sodium nitrite	1.0
218	Glutamic acid-5-butyl ester	1.0
		

TABLE 6. (Continued)

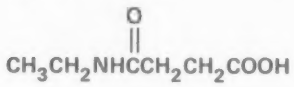
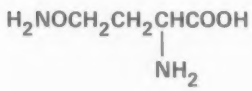
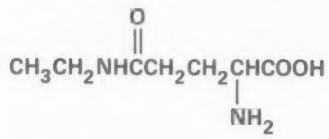
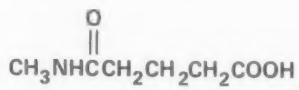
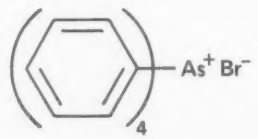
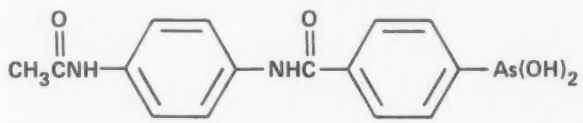
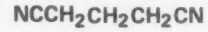
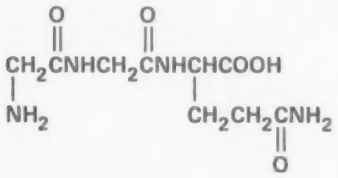
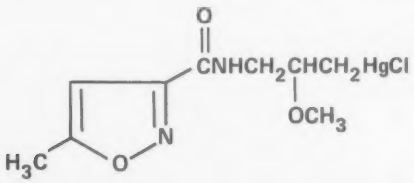
A Numbers	Compound	Concentrations Tested (mM)
219	*N*-Ethyl succinamate 	1.0
220	L-Canaline 	1.0
221	L-Glutamine, N-ethyl- 	1.0
222	*N*-Methyl glutaramic acid 	1.0 10.0
223	Arsonium tetraphenyl bromide 	1.0
224	Benzenearsonous acid, *p*-[(*p*-acetamidophenyl)carbamoyl]- 	1.0
225	Potassium arsenite (NSC-3060) 	1.0
226	Glutaronitrile (NSC-3807) 	1.0

TABLE 6. (Continued)

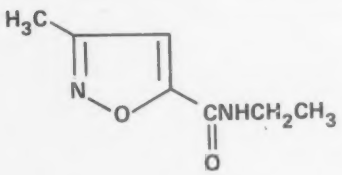
A Numbers	Compound	Concentrations Tested (mM)
227	Glycyl-glycyl-L-glutamine	1.0



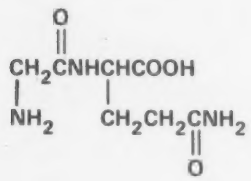
228	3-Isoxazolecarboxamide, *N*-[3-(chloromercuri)-2-methoxypropyl]-5-methyl (NSC-58764)	1.0



229	5-Isoxazolecarboxamide, *N*-ethyl-3-methyl (NSC-66023)	1.0



230	Glycyl-L-glutamine	1.0 10.0



231	Vanadyl sulfate, dihydrate (NSC-79532)	1.0

$VOSO_4 \cdot 2H_2O$

232	Butanoic acid, 2-amino-4-cyano-, (*S*)-	1.0 10.0

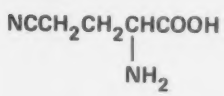


TABLE 6. (Continued)

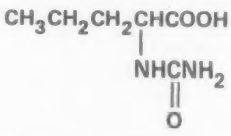
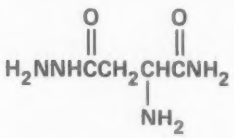
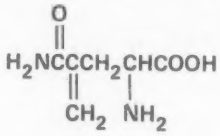
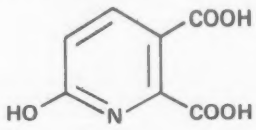
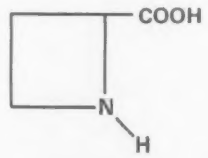
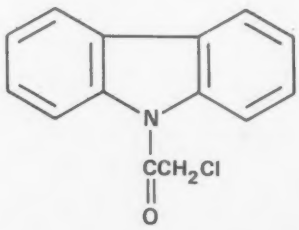
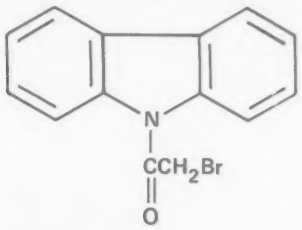
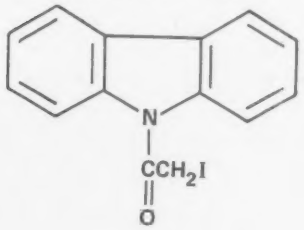
A Numbers	Compound	Concentrati Tested (mM)
233	Norvaline, *N*-carbamoyl-, DL- (NSC-144202)	1.0
		
234	Butanoic acid, 3,4-diamino-4-oxo-hydrazide, (±)-	1.0
		
235	L-Glutamine, 4-methylene-	5.0 10.0
		
236	2,3-Pyridinedicarboxylic acid, 6-hydroxy- (NSC-407242)	1.0
		
237	L-2-Azetidinecarboxylic acid (NSC-72471)	1.0
		
238	9*H*-Carbazole, 9-(chloroacetyl)-	1.0
		

TABLE 6. (Continued)

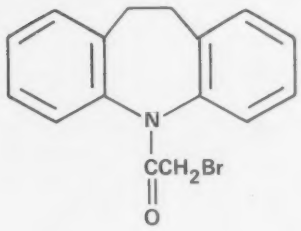
A Numbers	Compound	Concentrations Tested (mM)
239	9*H*-Carbazole, 9-(bromoacetyl)-	1.0



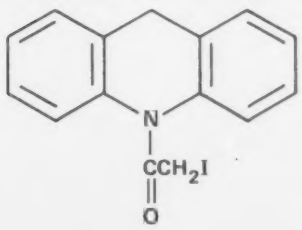
240	9*H*-Carbazole, 9-(iodoacetyl)-	1.0



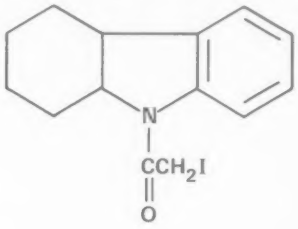
241	5*H*-Dibenz [*b*,*f*]azepine, 5-(bromoacetyl)-10,11-dihydro	1.0



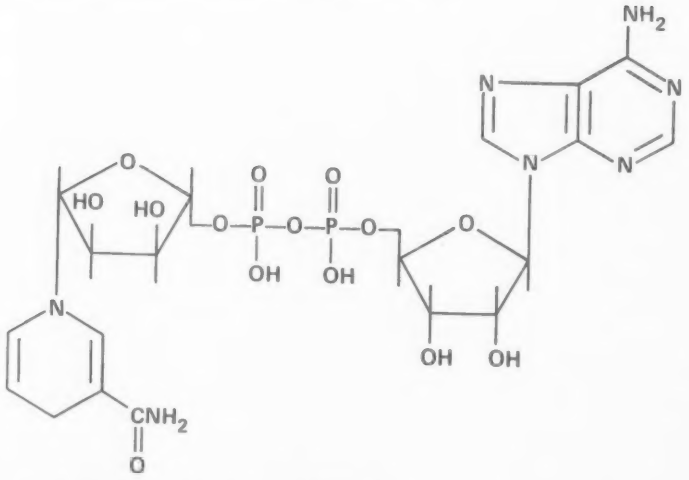
242	Acridine, 9,10-dihydro-10-(iodoacetyl)-	1.0



A Numbers	Compound	Concentrations Tested (mM)
243	1*H*-Carbazole, 2,3,4,9-tetrahydro-9-(iodoacetyl)-	1.0



244	β-Diphosphopyridine nucleotide (reduced form) (NADH)	1.0



245	Xanthosine-5′-monophosphoric acid (XMP)	1.0

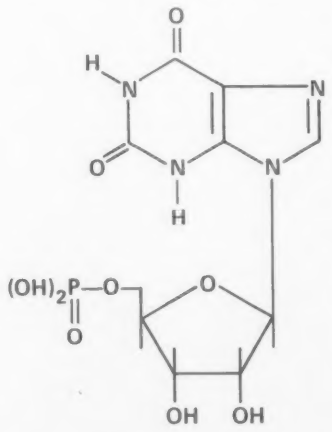


TABLE 6. (Continued)

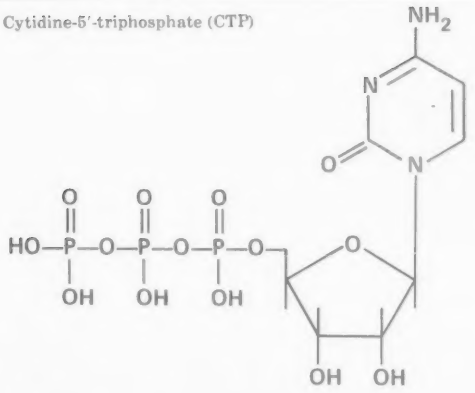
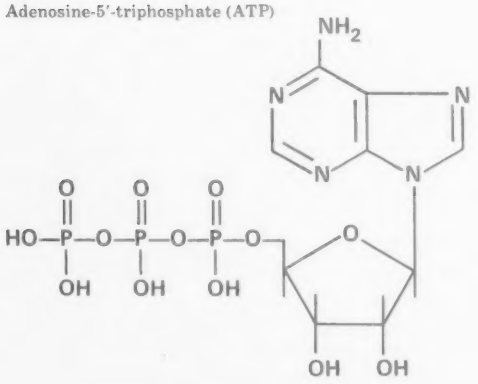
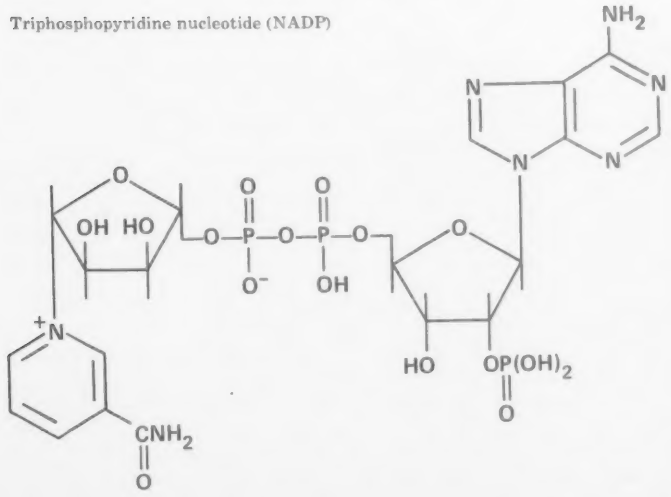
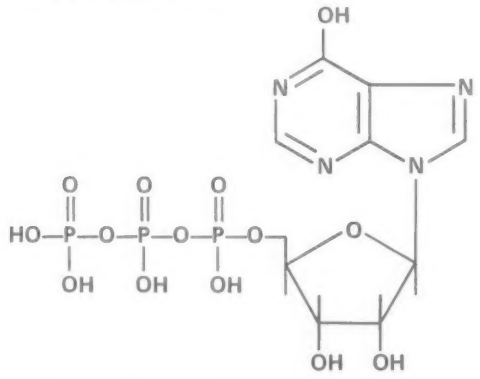
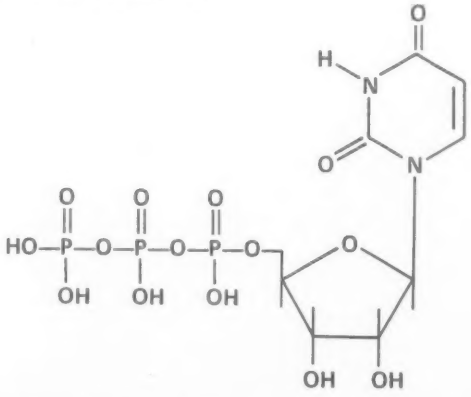
A Numbers	Compound	Concentrations Tested (mM)
246	Cytidine-5′-triphosphate (CTP) 	1.0
247	Adenosine-5′-triphosphate (ATP) 	1.0
248	Triphosphopyridine nucleotide (NADP) 	1.0

TABLE 6. (Continued)

A Numbers	Compound	Concentrations Tested (mM)
249	Inosine-5′-triphosphate (ITP)	1.0



250	Uridine-5′-triphosphate (UTP)	1.0



251	Thymidine-5′-triphosphate (TTP)	1.0

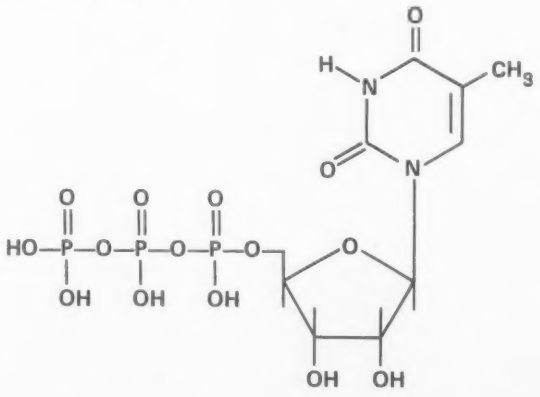


TABLE 6. (Continued)

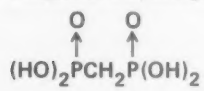
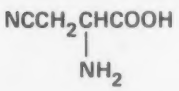
A Numbers	Compound	Concentrations Tested (mM)
252	Magnesium chloride $MgCl_2$	1.0
253	Barium acetate $Ba(OCOCH_3)_2$	1.0
254	Sodium chloride NaCl	1.0
255	Sodium iodide NaI	1.0
256	Sodium fluoride NaF	1.0
257	Sodium bromide NaBr	1.0
258	Ferric chloride $FeCl_3$	1.0
259	Beryllium sulfate $BeSO_4$	1.0
260	Strontium chloride $SrCl_2$	1.0
261	Phosphonic acid, methylenebis- 	1.0
262	Chromium chloride $CrCl_3 \cdot 6H_2O$	1.0
263	Zirconyl chloride $ZrOCl_2$	1.0
264	Alanine, 3-cyano- (NSC-529055) 	1.0

TABLE 6. (Continued)

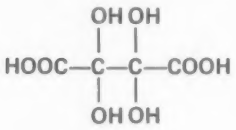
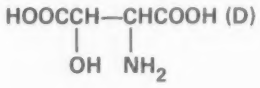
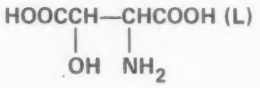
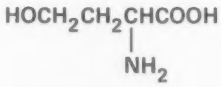
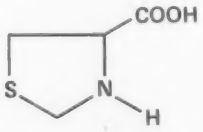
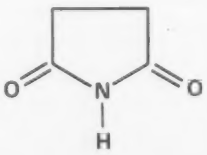
A Numbers	Compound	Concentrations Tested (mM)
265	Succinonitrile (NSC-4852) $NCCH_2CH_2CN$	1.0
266	Succinic acid, tetrahydroxy- (NSC-4647) 	1.0
267	Yttrium nitrate $Y(NO_3)_3$	1.0
268	Aspartic acid, 3-hydroxy-, D-erythro- (NSC-158204) 	1.0
269	Aspartic acid, 3-hydroxy-, L-erythro- (NSC-158205) 	1.0
270	L-Homoserine 	1.0
271	4-Thiazolidinecarboxylic acid 	1.0 5.0
272	Succinimide (NSC-11204) 	1.0 10.0

TABLE 6. (Continued)

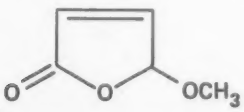
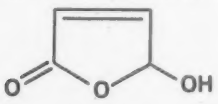
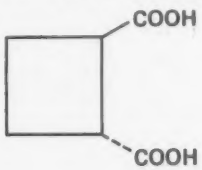
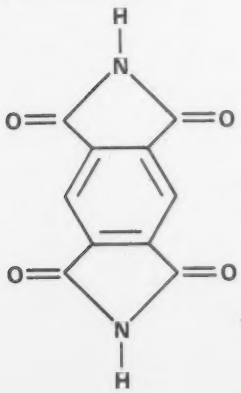
A Numbers	Compound	Concentrations Tested (mM)
273	2(5*H*)-Furanone, 5-chloro-	1.0
274	2(5*H*)-Furanone, 5-methoxy- 	1.0
275	2(5*H*)-Furanone, 5-hydroxy- 	1.0
276	*trans*-1,2-Cyclobutanedicarboxylic acid (NSC-527264) 	1.0
277	Pyromellitic diimide 	1.0

TABLE 6. (Continued)

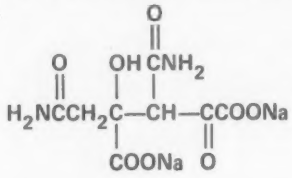
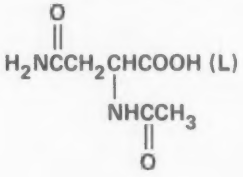
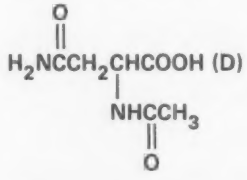
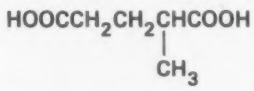
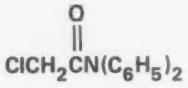
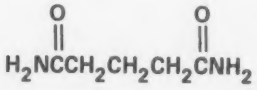
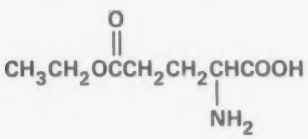
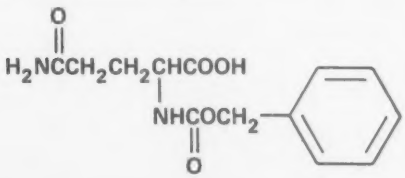
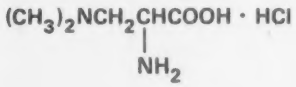
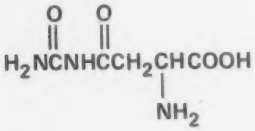
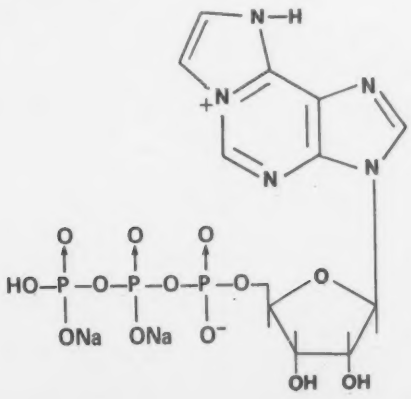
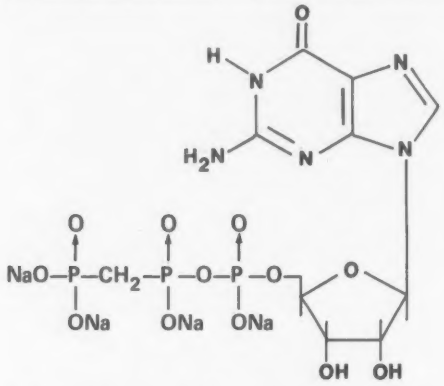
A Numbers	Compound	Concentrations Tested (mM)
278	Glutaric acid, 3-carbamoyl-2-(carbamoyl-methyl)-2-hydroxy-4-oxo-, disodium salt (DIMER OF α-KETOSUCCINAMIC ACID) (NSC-141661) 	1.0
279	L-Asparagine, N^2-acetyl- 	10.0
280	D-Asparagine, N^2-acetyl 	10.0
281	D-α-Methylglutamic acid 	1.0 10.0
283	Acetamide, 2-chloro-*N*,*N*-diphenyl 	1.0
284	Glutaric acid diamide 	1.0 10.0

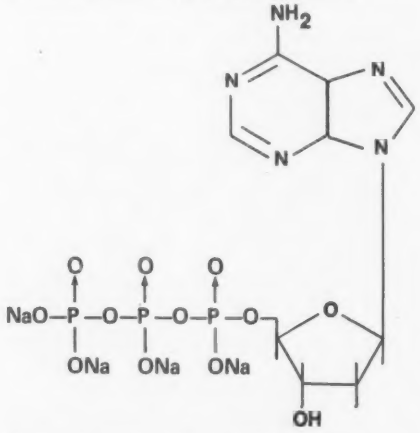
TABLE 6. (Continued)

A Numbers	Compound	Concentrations Tested (mM)
285	D-Glutamic acid 5-ethyl ester 	1.0 10.0
286	N^2-Carbobenzoxy-DL-glutamine 	1.0 10.0
287	DL-Alanine, 3-(dimethylamino)monohydrochloride DL-AZALEUCINE HCl 	1.0 5.0
288	*N*-carbamyl-L-asparagine 	1.0
289	1,N^6-Ethenoadenosine-5′-triphosphate (etheno ATP) 	1.0

A Numbers	Compound	Concentrations Tested (mM)
290	β,γ-methyleneguanosine-5′-triphosphate (β,γ-methylene GTP)	1.0



291	2′-Deoxyadenosine-5′-triphosphate (dATP)	1.0



292	Guanosine-5′-triphosphate (GTP)	1.0

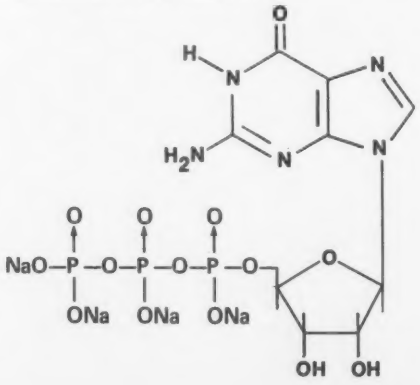
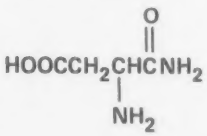


TABLE 6. (Continued)

A Numbers	Compound	Concentrations Tested (mM)
293	L-Isoasparagine 	1.0

Methyl-CCNU: Preclinical Toxicologic Evaluation of a Single Iv Infusion in Dogs and Monkeys[1,2]

U. Schaeppi,* R. W. Fleischman, R. S. Phelan, M. F. Ethier, and Y. K. Luthra[3,4]

SUMMARY

Methyl-CCNU, a compound with marked antitumor activity against the solid Lewis lung tumor in mice, was submitted to a preclinical pharmacologic evaluation. The toxicity of a single iv infusion was tested in 37 beagle dogs and 21 rhesus monkeys. The minimum lethal dose (LD) in dogs was 14 mg/kg and five of six dogs died within 7–10 days after treatment from hematopoietic toxicity with neutropenia, lymphopenia, anemia, and concomitant sepsis. Metaplasia of the intestinal epithelium also occurred. Thrombocytopenia was not observed. Dogs treated with 9.27–1.56 mg/kg exhibited reversible neutropenia and lymphopenia but survived without severe morbidity or histopathologic lesions. In monkeys, interstitial nephritis was the treatment-limiting toxicity and three of six monkeys treated with 45 or 30 mg/kg died, became moribund, or exhibited severe renal histopathologic lesions. One monkey treated with 45 mg/kg had degeneration of the testes. Survivors exhibited reversible toxicity and no histopathologic lesions. Treatment with doses as low as 7.5 mg/kg caused reversible neutropenia, lymphopenia, and anemia. The largest nontoxic dose for a single iv infusion was 3.12 mg/kg (62.40 mg/m^2) for the dog and 3.75 mg/kg (45 mg/m^2) for the monkey. These and earlier observations showed that methyl-CCNU had approximately one third the toxicity of CCNU. Methyl-CCNU also did not cause the delayed hepatic toxicity which is characteristic of CCNU treatment in the dog.

[Cancer Treat Rep 60:1559–1566, 1976]

Methyl-CCNU was synthesized by Johnston et al in 1966 (1). The compound produced weak antitumor activity in the L1210 leukemia system and was later found to have potent activity against the solid metastatic Lewis lung carcinoma (2). The present preclinical pharmacologic evaluation was performed prior to the use of methyl-CCNU in clinical trials.

METHODS

Experiments were performed on 37 beagle dogs and 21 rhesus monkeys in accordance with the procedures outlined by Prieur et al (3). Dogs and monkeys were submitted to a preclinical pharmacologic evaluation that included physical examinations, hematologic and blood chemical determinations, necropsies, and histopathologic evaluations, as described by Schaeppi et al (4). Blood determinations included hematocrit, hemoglobin, cell counts of red blood cells (rbcs), white blood cells (wbcs), thrombocytes, white blood cell differential, and reticulocytes, blood urea nitrogen (BUN), blood glucose, alkaline phosphatase (AP), serum glutamic pyruvic

[1]Supported by contract NIH-70-2055 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methyl-CCNU: NSC-95441; CAS reg. No. 33073–59–5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-. Lots BVL-110,970A, BVL-110,970B, BVL-71-217, and BVL-72-250 in vials of 100 and 200 mg were supplied by the Drug Development Branch, Drug Research and Development Program (DR&DP), DCT, NCI.

[3]Mason Research Institute, Worcester, Mass.

[4]We thank our project officers, Dr. D. A. Cooney and Mrs. R. D. Davis, from the Laboratory of Toxicology, Developmental Therapeutics Program, DCT, NCI, for directions received in the performance of this study; Dr. V. Ilievski and Dr. H. Rosenkrantz from Mason Research Institute, for clinical veterinary services and for supervision of clinical blood determinations respectively; and Dr. M. D. Walker from the Baltimore Cancer Research Center, DCT, NCI, for instruction and assistance in the performance of iv infusions.

*_Reprint requests to:_ Dr. Ulrich Schaeppi, Toxicology/Pathology, CIBA-GEIGY Ltd, CH-4002, Basle, Switzerland.

TABLE 1.—Methyl-CCNU toxicity following a single iv infusion in dogs*

	Acute toxicity dose levels: mg/kg (mg/m²)					Delayed toxicity dose levels (> 3 wks after treatment): mg/kg (mg/m²)				
	14 (280.0)	9.37 (187.4)	6.25 (125.0)	3.12 (62.40)	1.56 (31.20)	14 (280)	9.37 (187.4)	6.25 (125.0)	3.12 (62.40)	1.56 (31.20)
No. of dogs tested	6	10	7	7	7	1	10	7	7	7
Clinical observations										
Mortality (Days of death, 7–10)	5/6									
Emesis	2/6									
Diarrhea	5/6	1/10								
Pyrexia	4/6	1/10								
Abdominal tenderness		1/10	2/7							
Deteriorated condition	6/6									
Weight loss	6/6	1/10								
Blood determinations										
Anemia	6/6	6/10								
Neutropenia	6/6	10/10	4/7	1/7	1/7					
Lymphopenia	5/6	2/10	1/7	1/7	1/7					
Monocytosis	1/6									
Reticulocytopenia	6/6	3/10								
Azotemia	2/6	—								
BSP retention	NT†	2/10								
AP	6/6	6/10	2/7	1/7		1/1	2/10	1/7	2/7	
SGOT	3/3									
SGPT	2/6	2/10		1/7		1/1	1/10			
Derangements of Na^+, K^+, or Cl^-	6/6									
Pathology										
Necropsy performed	← Days 7–10 →					← Days 50–368 →				
No. necropsied	5	0	0	0	0	1	10	7	7	7
Bone marrow hypocellularity	5/5									
Lymphoid tissue hypocellularity	5/5									
Septicemia	5/5									
Colitis or proctitis	2/5									
Hemorrhages	4/5									

*No. of dogs with toxicity/No. tested. No entry = no toxicity. 2 dogs treated with the control vehicle (propylene glycol/ethanol) and 2 dogs treated with Emulphor exhibited no abnormal deviations of the above parameters.

†NT = not tested.

transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), sodium (Na^+), potassium (K^+), chlorides (Cl^-), calcium (Ca^{++}), CO_2, bromsulphalein (BSP) retention, bilirubin, and prothrombin time. Plasma and urine hemoglobin was determined according to Kohn and Kelley (5).

Dogs and monkeys were infused iv with a single dose of methyl-CCNU. Tables 1 and 2 present information on doses and numbers of animals tested for each dose level.

All animals were killed when they became moribund or after a posttreatment observation period of approximately 50 days in dogs and 100 days in monkeys. In addition, three dogs treated with 9.37 mg/kg and one each at dose levels of 6.25, 3.12, and 1.56 mg/kg were necropsied 163–368 days after treatment. Hematoxylin and eosin sections were prepared from approximately 47 organs and tissues and were examined histologically.

Infusions were performed under aseptic conditions by means of spaghetti tubing 30 TW (E. L. Nichols Co, Inc, West Boylston, Mass) temporarily inserted into the cephalic or saphenous vein.

Toxicity studies were performed with methyl-CCNU (200 and 100 mg/vial) formulated in three different vehicles as reported in detail elsewhere (6).

Formulation: methyl-CCNU (35.7 mg/ml) in 80% propylene glycol and 20% ethanol.—Methyl-CCNU (100 mg) was dissolved in 0.56 ml of absolute ethanol. Propylene glycol (2.24 ml) was then added, resulting in a final concentration of methyl-CCNU of 35.7 mg/ml. Iv infusions were performed within 15–30 minutes.

Formulation: methyl-CCNU (5 mg/ml) in 5% in-

TABLE 2.—Methyl-CCNU toxicity following a single iv infusion in rhesus monkeys*

	Acute toxicity dose levels: mg/kg (mg/m²)						Delayed toxicity dose levels (> 3 wks after treatment): mg/kg (mg/m²)					
	45 (540)	30 (360)	15 (180)	7.5 (90)	3.75 (45)	1.875 (23)	45 (540)	30 (360)	15 (180)	7.5 (90)	3.75 (45)	1.875 (23)
No. of monkeys tested	2	5	5	5	2	2	1	5	5	5	2	2
Clinical observations												
Mortality or severe morbidity	1/2						1/1	1/5				
Emesis	1/2	4/5										
Diarrhea	1/2		1/5									
Deteriorated condition	1/2						1/1					
Abdominal pain	1/2											
Weight loss	1/2						1/1	2/5	2/5	1/5		
Blood determinations												
Anemia	2/2	5/5	5/5				1/1	5/5	3/5	2/5		
Neutropenia	2/2	5/5	5/5				1/1	5/5	4/5	1/5		
Lymphopenia	2/2	5/5					1/1	1/5	3/5			
Reticulocytopenia	1/2	3/5										
Thrombocytopenia												
BUN	1/2	1/5					1/1	2/5				
Derangements of Na^+, K^+, or Cl^-	1/2	5/5					1/1	4/5				
Ca^{++} (↓/↑)							1/1	1/5				
AP (↑)		2/5	2/5	1/5		1/2		2/5	3/5		1/2	1/2
SGPT (↑)		3/5	1/5									
SGOT (↑)		3/5	1/5					4/5	2/5			
Pathology												
Day of necropsy	8						75	100	100	100	100	100
Renal toxicity							1/1	2/5				
Bone marrow hypocellularity	1/1											
Lymphoid tissue hypocellularity	1/1											
Intestines, epithelial metaplasia	1/1											
Testicular degeneration							1/1					

*No. of monkeys with toxicity/No. tested. No entry = no toxicity. 4 monkeys treated with the control vehicles exhibited no deviations of the above parameters.

tralipid, 5% ethanol, and 90% saline.—Methyl-CCNU (100 mg/ml) in absolute ethanol was mixed with 19 ml of intralipid[5] and saline by slowly adding the methyl-CCNU solution with a syringe and 27-gauge needle to intralipid-saline that was kept in swirling motion by means of a vortex mixer. Iv infusions were performed within 9–19 minutes.

Formulation: methyl-CCNU (2.5 mg/ml) in 5% Emulphor, 5% ethanol, and 90% saline.—Methyl-CCNU (100 mg) dissolved in 4 ml of Emulphor[6]-absolute ethanol (50% vol/vol) was added to 16 ml of saline, resulting in a final concentration of methyl-CCNU of 2.5 mg/ml. Iv infusions were administered within 42–100 minutes.

Iv administration of Emulphor in dogs caused histamine release and a subsequent transient decrease of blood pressure (7).[7] Following histamine release, the subsequent iv administration of Emulphor produced no further important hypotension. To minimize the combination of toxicity of histamine and toxicity of methyl-CCNU, infusions of methyl-CCNU in Emulphor were consistently started 120 minutes after a priming injection with 0.2 ml/kg of Emulphor as recommended by Dr. M. Walker of the

[5]Intralipid (Vitrum, Stockholm, Sweden), a 10% soyabean oil emulsion also containing 1.2% phospholipids, 2.25% glycerol, and water for injection, was supplied in 20-ml vials. The product has a milk-like appearance.

[6]Emulphor-ethanol (ca 50% vol/vol) was supplied in 4-ml ampules. Emulphorethanol was diluted with saline to a final concentration of 5% Emulphor (surfactant), 5% ethanol, and 90% saline.

[7]Peck HM, Baier JE, McKinney SE, et al. Emulphor EL-620 vehicle toxicologic evaluation. Report from Merck, Sharp and Dohme Research Laboratories, Mar 23, 1972, on revised data from Oct 22, 1959.

Schmidt LH, Sullivan RW, Lang LF, et al. The reactions of the rhesus monkey and dog to Emulphor EL-719. The Christ Hospital Institute of Medical Research, Cincinnati, Ohio. Report on studies carried out under contract SA-43-ph-1905, Oct 30, 1961.

Baltimore Cancer Research Center.[8] The same procedure had been used in earlier studies with iv infusions of CCNU (7).

The toxic dose high was defined as one half of the minimum lethal dose (LD). The toxic dose low was defined as the smallest dose which produced toxicity; doubling of this dose had to be nonlethal (3). A detailed description of all features of this work was presented in previous reports to the National Cancer Institute.[9]

RESULTS

Five of six dogs treated with a single iv infusion of methyl-CCNU (14 mg/kg) died or became moribund within 7–10 days after treatment (table 1). The first clinical signs of toxicity appeared 7 days after treatment and included pyrexia (four of six), various signs of septicemia, and hemorrhagic diarrhea (five of six). All six dogs developed a severe neutropenia and lymphopenia. Neutrophils and lymphocytes started to decrease on Day 4 and reached a nadir on Day 7. Neutropenia was usually more severe than lymphopenia. In dogs that died, the neutrophils decreased to 87 cells/mm^3 or less or they disappeared entirely from the peripheral circulation. Thrombocytopenia did not occur. The necropsy and histopathologic evaluation of dogs with severe acute morbidity showed severe hypocellularity of the bone marrow and lymphoid tissues (five of five), severe necrotic tonsillitis (five of five), interdigital cellulitis (two of five), bacteremia (two of five), fibrino-hemorrhagic pneumonia (two of five), multiple hemorrhages (four of five), and colitis or proctitis (two of five). The one dog surviving treatment with 14 mg/kg developed a neutropenia of 361 cells/mm^3 with no pyrexia or sepsis. The dog had a reversible elevation of SGPT with maximum values on Day 22 (fig 1) and concomitant elevation of AP. The blood values regressed thereafter to within normal limits and the dog was killed in good general condition on Day 101.

The other dogs treated with 9.37, 6.25, 3.12, or 1.56 mg/kg survived. One of ten dogs treated with 9.37 mg/kg had diarrhea on Days 3 and 9–13 and pyrexia on Days 7–9 and two of seven dogs treated with 6.25 mg/kg developed reversible abdominal tenderness.

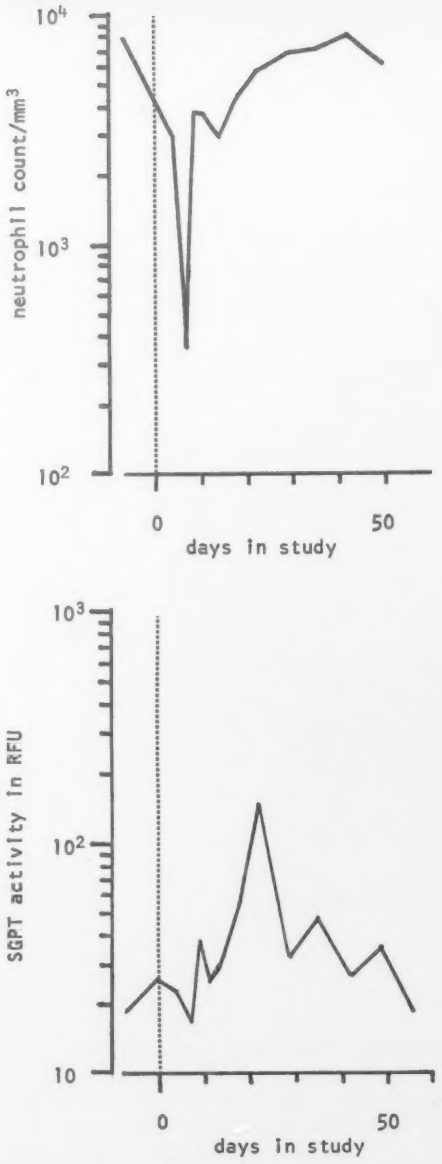


FIGURE 1.—Peripheral neutrophil count and SGPT activity in 1 of 6 dogs surviving iv infusion of methyl-CCNU (14 mg/kg).

Dogs treated with 9.37–1.56 mg/kg developed dose-related neutropenia (fig 2) and lymphopenia and those treated with 9.37–3.12 mg/kg had occasional mild elevations of AP and SGPT.

The surviving dogs were killed in good general condition from Days 49 to 368. The histopathologic evaluation showed no gross or microscopic lesions.

[8]Walker M. Preliminary evaluation of EL-620. Memorandum from the Acting Chief, Baltimore Cancer Research Center, DCT, NCI, to Chief, Drug Development Branch, DR&DP, DCT, NCI, July 24, 1972.

[9]Reports on the toxicity of methyl-CCNU (*see* Appendix) were filed with and are available from the US Department of Commerce National Technical Information Service, Springfield, Va 22151.

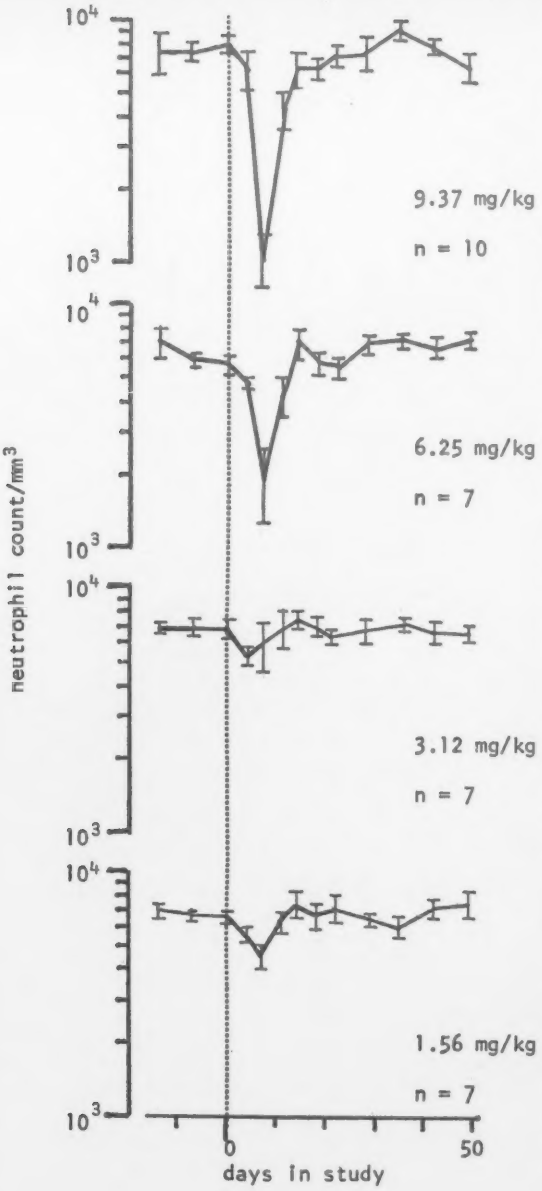


FIGURE 2.—Peripheral neutrophil count after iv infusion of methyl-CCNU in dogs. Mean values ± standard error, n = number of dogs tested at each dose level. Treatment at all doses failed to cause significant changes in SGPT activity.

Methyl-CCNU infused in three different formulations (80% propylene glycol/20% ethanol, 5% intralipid/5% ethanol/90% saline, or 5% Emulphor/5% ethanol/90% saline) caused qualitatively the same overall toxic effects with the exception of additional hemolysis caused by propylene glycol and hypotensive effects caused by Emulphor in the dog.[10] Formulations in Emulphor were administered within 65–72 minutes to reduce hypotensive effects. This administration, which was more protracted than for the other two formulations, caused less frequent mortality (*see* table 3).

One of the two monkeys infused with 45 mg/kg was killed with severe acute morbidity on Day 8 and the other died from uremia on Day 75. The monkey with severe acute toxicity developed diarrhea after Day 5, preterminal neutropenia of 169 cells/mm^3 without pyrexia, a BUN level of 85 mg/100 ml, and various serum electrolyte derangements. The histopathologic evaluation showed moderate hypocellularity of the bone marrow and lymphoid tissues, epithelial metaplasia of the esophagus and small and large intestines, and focal ulcerative colitis. We believed that the intestinal lesions had caused the poor general condition and that treatment-induced activation of a latent Shigellosis could have contributed to severe morbidity. The other top-dose monkey died on Day 75 with preterminal uremia. The histopathologic evaluation revealed a glomerulonephritis and tubular dysplasia, a uremic pneumonitis, hypocellularity of the lymphoid tissues, and focal degeneration of the testes.

Monkeys infused with 30 mg/kg responded with elevation of BUN (two of five) and derangement of serum electrolytes (five of five). One animal killed in poor general condition on Day 100 had a preterminal uremia, a severe interstitial nephritis, and tubular dysplasia. Another monkey killed on Day 100 in apparently good general condition had a marked chronic interstitial nephritis. The other monkeys treated with 30 mg/kg and those treated with 15–1.87 mg/kg survived and necropsies performed 100 days after treatment showed no histopathologic lesions.

Dose-related neutropenia occurred in monkeys treated with 45 (two of two), 30 (five of five), 15 (five of five), and 7.5 (two of five) mg/kg (table 2). Lymphopenia was equal in severity to neutropenia. In survivors, leukopenia disappeared within 4–5 weeks following treatment (fig 3).

Treatment caused occasional non-dose-related reversible elevation of AP and transaminase activities. SGPT activity, in particular, was not increased more than twofold above control values. SGOT con-

[10]Methyl-CCNU, 2.5 mg/ml in Emulphor, was chosen for subsequent clinical trials because severe hypotension was restricted to the dog and because the procedure for formulation with intralipid appeared to be too complicated for routine treatments.

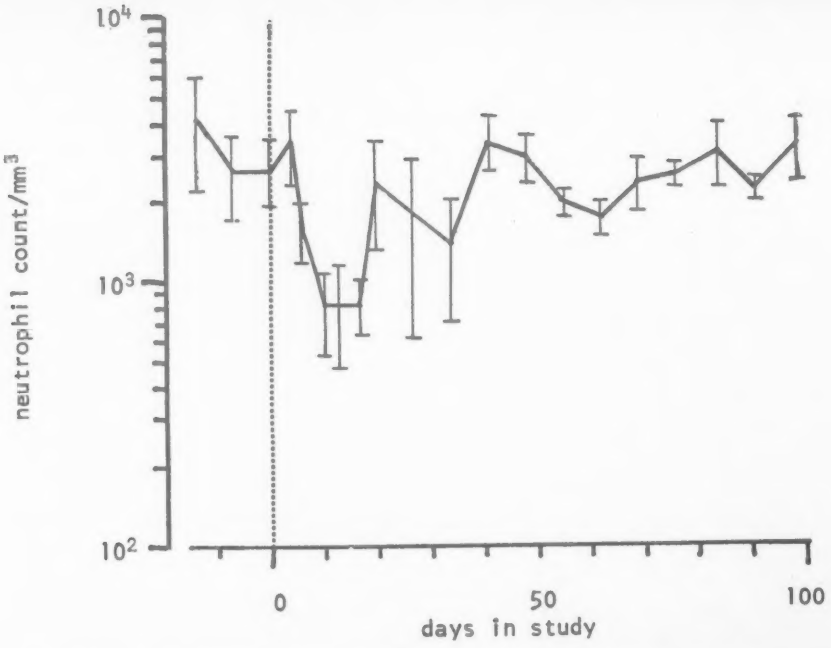


FIGURE 3.—Peripheral neutrophil count in rhesus monkeys treated with iv infusion of methyl-CCNU (30 mg/kg). Mean values ± standard error from 5 monkeys. The blood determination on Day 0 was performed before the iv infusion.

TABLE 3.—Mortality of 6 dogs infused with methyl-CCNU (14 mg/kg) using different vehicles and durations of iv infusion

Vehicle	Duration of infusion (min)	Mortality rate
20% propylene glycol/80% ethanol	21–27	2/2
5% intralipid/5% ethanol/90% saline	19–23	2/2
5% Emulphor/5% ethanol/90% saline	65–72	1/2

sistently exceeded SGPT activity indicating a nonhepatic origin of transaminase elevation.

DISCUSSION

Nitrosoureas, including BCNU, CCNU, and methyl-CCNU, have shown a similar drug-disposition pattern. Iv injected parent compound was rapidly metabolized and disappeared from the plasma with a half-life of several minutes. Metabolites were excreted into bile and urine but were eliminated primarily via the kidneys because of enterohepatic cycling. For the metabolites, the half-life for clearance from the plasma was several hours (8–10). The active form for anticancer and toxic activity of the individual nitrosoureas has not been identified, but it is not necessarily the same compound (10).

For methyl-CCNU, the treatment-limiting toxicity was hematopoietic toxicity for the dog, with mortality occurring at 14 mg/kg (280 mg/m^2). Hematopoietic toxicity for the monkey was not as severe as it was for the dog, and treatment-limiting toxicity consisted of irreversible progressive renal lesions that caused delayed mortality, with an LD of 30 mg/kg (360 mg/m^2) (table 4). The minimum LD for a single iv infusion of methyl-CCNU was 2.8 times greater in dogs and 3 times greater in monkeys than for CCNU (7). Doses smaller than the LD produced reversible toxicity. The minimum dose of methyl-CCNU which caused toxicity to organs and tissues increased for the dog in the following order: bone marrow and lymphoid tissue, liver, intestines, kidney, and testes. For the monkey the order was: bone marrow and lymphoid tissue, kidney, intestines, testes, and liver (table 5). The highest nontoxic dose was 3.12 mg/kg (62.40 mg/m^2) for the dog and 3.75 mg/kg (45 mg/m^2) for the monkey.

The present observation that the rate of mortality paralleled the speed of infusion and previous observations that plasma levels of nitrosoureas and metabolites declined rapidly following iv injection (8–10) would then indicate that toxicity parallels peak plasma levels.

In man, methyl-CCNU, like CCNU, had qualita-

TABLE 4.—Toxicity ranking in dogs and monkeys treated with a single iv infusion of methyl-CCNU

	Dogs			Monkeys		
	mg/kg	mg/m²	M/C*	mg/kg	mg/m²	M/C*
Highest nontoxic dose	1.56	31.2	2.5	3.75	45	3
Toxic dose low	3.12†	62.4	2.5	7.5	90	3
Toxic dose high	7	140	2.8	15	180	3
Lethal dose	14	280	2.8	30	360	1.5–3

*M/C = toxicity factors for comparison of defined toxic doses of CCNU with those of methyl-CCNU. Multiplication of defined toxic doses for CCNU (7) with the toxicity factor indicates the corresponding toxic dose for methyl-CCNU.

†No important toxicity observed.

TABLE 5.—Minimum toxic doses for organs and body systems in dogs and monkeys treated with a single iv infusion of methyl-CCNU

	Dogs			Monkeys		
	mg/kg	mg/m²	M/C*	mg/kg	mg/m²	M/C*
Bone marrow	1.56	31.2	1.2	7.5	90	3
Lymphoid tissues	1.56	31.2	0.6	7.5	90	3
Kidneys	> 14	> 280		30	180	3
Liver	9.37	187.4	3.8	> 45	> 540	
Testes	> 14	> 280		45	540	4.5
Intestinal toxicity	14	280	2.8	45	540	1.5

*M/C = toxicity factors for comparison of CCNU with methyl-CCNU with respect to minimum toxic doses in organs and body systems. Multiplication of the toxic dose for CCNU (7) with the toxicity factor indicates an equivalent toxic dose for methyl-CCNU.

tively somewhat different toxic effects than in the large-animal screen of dogs and monkeys. Humans treated with nitrosoureas responded with an early and delayed leukopenia and thrombocytopenia (11). In contrast, thrombocytopenia was not an important toxic manifestation in monkeys and dogs and the delayed phase of bone marrow toxicity was relatively mild in the monkey and did not occur in the dog.

APPENDIX (Reports on the Toxicity of Methyl-CCNU)

1. SCHAEPPI U, FLEISCHMAN RW, ROSENKRANTZ H, ET AL. Methyl-CCNU (NSC-95441): toxicity of a single i.v. infusion in dogs. Vehicle: intralipid 95%-ethanol 5%. MRI-CC03-72-11 (Apr 7, 1972).
2. FLEISCHMAN RW, SCHAEPPI U, ROSENKRANTZ H, ET AL. Methyl-CCNU (NSC-95441): toxicity of a single i.v. infusion in dogs. Vehicle: 20% absolute alcohol-80% propylene glycol. MRI-CC04-72-14 (May 5, 1972).
3. SCHAEPPI U, FLEISCHMAN RW, ILIEVSKI V, ET AL. Methyl-CCNU (NSC-95441): toxicity in dogs treated with 3.12 mg/kg once per week for 2 and 3 Rx. Vehicle: 20% absolute alcohol, 80% propylene glycol. MRI-CC05-72-23 (June 16, 1972).
4. SCHAEPPI U, PHELAN RS, FLEISCHMAN RW, ET AL. Methyl-CCNU (NSC-95441): toxicity of 1 single i.v. infusion in rhesus monkeys. Vehicle: propylene glycol 80%, ethanol 20%. MRI-CC07-72-39 (Sept 8, 1972).
5. SCHAEPPI U, PHELAN RS, COONEY DA, ET AL. Emulphor (EL-620) vehicle: blood pressure effects of i.v. infusion in dogs and rhesus monkeys. MRI-CC09-72-42 (Sept 29, 1972).
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evaluation of intravenous injection in dogs, monkeys and man. Toxicol Appl Pharmacol 25:230–241, 1973.

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7. SCHAEPPI U, FLEISCHMAN RW, PHELAN RS, ET AL. CCNU (NSC-79037): preclinical toxicologic evaluation of a single intravenous infusion in dogs and monkeys. Cancer Chemother Rep Part 3, vol 5(1):53–64, 1974.
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Inhibitors of Hypoxanthine Metabolism in Ehrlich Ascites Tumor Cells In Vitro[1]

Camilla M. Smith, George Zombor, and J. Frank Henderson[2,*]

SUMMARY

One hundred and sixty-one purine analogs and derivatives were tested for their ability to inhibit ten parameters of purine metabolism in Ehrlich ascites tumor cells incubated in vitro with radioactive hypoxanthine. Sixty-seven compounds were inhibitory against at least one parameter and 30 were inhibitory against two or more.

[Cancer Treat Rep 60:1567–1584, 1976]

As part of an on-going biochemical screening program to obtain preliminary indications of biochemical activity of purine analogs and derivatives (1–7), we have searched for inhibitors of purine ribonucleotide biosynthesis and interconversion. The methods used permit the determination of the effect of test compounds on the apparent rates of the following enzymes or processes of purine ribonucleotide metabolism in intact Ehrlich ascites tumor cells incubated in vitro: (*a*) hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); (*b*) adenylosuccinate synthetase (EC 6.3.4.4) plus adenylosuccinate lyase (EC 4.3.2.2); (*c*) conversion of adenosine monophosphate (AMP) to adenosine diphosphate (ADP); (*d*) conversion of ADP to adenosine triphosphate (ATP); (*e*) inosinate (IMP) dehydrogenase (EC 1.2.1.14); (*f*) guanosine monophosphate (GMP) synthetase (EC 6.3.5.2); (*g*) conversion of GMP to guanosine diphosphate (GDP); and (*h*) conversion of GDP to guanosine triphosphate (GTP). In addition, the effects of inhibitors on the ratios ATP:ADP + AMP and GTP:GDP + GMP also are reported. The latter parameters are used mainly as indicators of the effects of compounds on energy metabolism, since such effects would necessarily affect other aspects of nucleotide metabolism as well.

We have previously reported studies of the effects of 92 purine analogs and derivatives in this system (7); here, studies of an additional 161 compounds are reported.

MATERIALS AND METHODS

Hypoxanthine labeled at the 8 position with ^{14}C (51 millicuries/mmol, 98% pure) was purchased from Schwarz BioResearch. Purine ribonucleotides were purchased from Sigma Chemical Co, Fischer's medium from Grand Island Biological Co, and polyethyleneiminecellulose thin-layer chromatography sheets on Mylar film from J. T. Baker Co.

Ehrlich ascites tumor cells were maintained by weekly ip injection of 5–6 × 10^6 cells in Ha/ICR mice from our own colony or from the A. R. Schmidt Co, Madison, Wis. For incubation in vitro, mice were killed and tumor cells removed and diluted at room temperature in Fischer's medium modified to contain 25 mM of phosphate buffer (pH 7.4) without bicarbonate. The cells were collected by centrifugation at room temperature and diluted in the modified Fischer's medium to make a 2.5% suspension by volume. Eighty μl of tumor cell suspension, 5 μl of medium, and 10 μl of test compounds (1 mM, final concentration) were then incubated in small plastic tubes (10 × 75 mm, Falcon Plastics) for 20 minutes at 37°C, with shaking at 120 oscillations/minute using air as the gas phase. Five μl of [^{14}C]hypoxanthine was then added (0.1 mM, final concentration) and the incubation was continued. After 60 minutes, 5 μl of 4.2 M perchloric acid was added to each tube, and the cell extract was neutralized by the addition of 5 μl of 4.42 M KOH. Samples were chilled and centrifuged and 10 μl of the supernatant was taken for chromatography studies. Purine ribonucleotides

[1]Supported by the National Cancer Institute of Canada.

[2]University of Alberta Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, Edmonton, Alberta, Canada.

[*]*Reprint requests to:* J. Frank Henderson, PhD, McEachern Laboratory, University of Alberta Cancer Research Unit, Edmonton, Alberta, Canada T6G 2H7.

(ATP, nicotinamide adenine dinucleotide [NAD], AMP, ADP, IMP, xanthosine monophosphate [XMP], GMP, GDP, GTP) were separated by chromatography on polyethyleneiminecellulose according to the method of Crabtree and Henderson (8) and the radioactivity in each compound was measured.

The apparent rate of each reaction or process of purine ribonucleotide metabolism was then calculated from the amount of radioactivity that had passed through that reaction or process. Percent inhibition was calculated by comparing the apparent rate of each reaction or process in the presence of test compound with that in control cells. Each result has been corrected for any inhibition of preceding reactions that may have occurred. The mathematic basis and the computer programs used for these calculations have been published elsewhere (9). Results are averages of duplicate tests. Average variation of individual analyses from the mean was < 7%.

RESULTS AND DISCUSSION

Table A lists the 161 compounds tested in this study and table 1 shows the percent inhibition each produced on the eight enzymes and two other parameters of purine metabolism tested. Sixty-seven compounds inhibited at least one process and 30 inhibited two or more.

Hypoxanthine Phosphoribosyltransferase

Thirty-six compounds produced > 50% inhibition of nucleotide formation from [^{14}C]hypoxanthine; of these, 16 produced > 75% inhibition. These 36 compounds are listed in table 2 in order of their inhibitory activities. Only three of the active compounds were unsubstituted at the 9 position and two of these were potent inhibitors of energy metabolism. Fourteen compounds were ribonucleosides, five were 2′-deoxyribonucleosides, and two were 3′-deoxyribonucleosides. Other substituents at the 9 position included furanone, tetrahydrofuryl, tetrahydropyranyl, and methanol groups. Most compounds contained purine bases but 11 pyrrolo(2,3-*d*)pyrimidines were also active. A wide variety of substituents at the 6 position or equivalent were compatible with activity, and three pyrrolo(2,3-*d*)pyrimidines substituted at the 5 position (equivalent to the purine 7 position) also were active.

Detailed studies of biochemical effects and growth-inhibitory properties of NSC-107519 and related compounds (10) and of NSC-144667, NSC-99930, and related compounds (11) have been reported elsewhere.

Adenylosuccinate Synthetase Plus Adenylosuccinate Lyase

Only nine compounds were at all inhibitory to these enzymes and only one produced > 50% inhibition (table 3). Seven of these compounds also inhibited energy metabolism, as indicated by the ATP:ADP + AMP and GTP:GDP + GMP ratios; in these cases inhibition of adenylosuccinate synthetase plus adenylosuccinate lyase may be indirect. The most specific compounds, NSC-124154 and NSC-132292, both are adenosine analogs or derivatives.

Conversion of AMP to ADP

This reaction can be catalyzed by adenylate kinase and by certain other nucleotide kinases, but the exact nature of this process under the conditions used here has not been determined. Only two compounds were at all active: NSC-145391 produced 45.3% inhibition and NSC-145067 produced 40.0% inhibition. Both are potent inhibitors of energy metabolism, and therefore their effects on the conversion of AMP to ADP probably are indirect.

Conversion of ADP to ATP

Only five compounds were at all inhibitory to this process and only two produced > 50% inhibition (table 4). All of these compounds are potent inhibitors of energy metabolism.

Inosinate Dehydrogenase

This reaction was inhibited by 21 compounds, of which six produced > 50% inhibition (table 5). Inhibitory compounds included purine, pyrrolo(2,3-*d*)pyrimidine, pyrazolo(3,4-*b*)pyridine, pyrazolo(3,4-*d*)pyrimidine, and indazole derivatives. Only four of these compounds were not nucleoside derivatives or analogs, but a wide range of ring substituents were compatible with activity. A detailed study of biochemical effects and growth-inhibitory properties of NSC-112521 and related compounds has been published (12).

Guanylate Synthetase

Only five compounds were at all inhibitory to this enzyme and none produced > 50% inhibition (table 6). Only NSC-116092 was completely specific for this reaction; the rest inhibited total nucleotide synthesis from [^{14}C]hypoxanthine, energy metabolism, or both.

Conversion of GMP to GDP

Two compounds inhibited this process: NSC-145391 produced 44.3% inhibition and NSC-145067 produced 38.5% inhibition. Both are potent inhibitors of energy metabolism and their effect on the conversion of GMP to GDP therefore probably is indirect.

Conversion of GDP to GTP

As in the case of inhibitors of ADP to ATP, the five inhibitors of the conversion of GDP to GTP (table 7) also were potent inhibitors of energy metabolism and several other reactions as well.

ATP:ADP + AMP Ratio

This ratio is an index of good energy metabolism and 28 compounds reduced it to < 50% of the control value; of these, ten produced > 75% inhibition (table 8). Two compounds, NSC-145391 and NSC-145067, were particularly potent inhibitors of energy metabolism and as a consequence caused apparent inhibition of most of the other parameters of purine metabolism that were measured. Three compounds were not substituted at the 9 or equivalent position; the rest had ribose or other sugars, methanol, or tetrahydro-thienyl groups at this position. Both purine and other ring systems were compatible with activity. Three compounds had an unsubstituted amino group at the 6 position or equivalent, ten had alkylamino or aralkyl groups, six had alkylthio group, and four had a chloro or fluoro group.

GTP:GDP + GMP Ratio

Twenty-one compounds reduced this ratio to 50% of the control value or less and seven produced > 75% inhibition (table 9). Five are not nucleosides and are not otherwise substituted at the 9 or equivalent position. A variety of substituents are permitted at this position however, as well as at the 6 position. Compounds representing several ring systems in addition to purine were among the active compounds.

Inhibition of Two or More Reactions

Of the 67 different compounds listed in tables 2–9, two inhibited eight systems, one inhibited seven, two inhibited six, four inhibited four, seven inhibited three, and 14 inhibited two. These inhibitors of multiple processes are listed in table 10. Fourteen compounds inhibited only hypoxanthine phosphoribosyltransferase, ten inhibited only inosinate dehydrogenase, one inhibited only guanylate synthetase, nine inhibited only the ATP:ADP + AMP ratio, and seven inhibited only the GTP:GDP + GTP ratio.

TABLE A. -- List of compounds tested

NSC No.	CRU No.	Compound	Molecular formula	Source*
7841	65	6-Purinethiol, 9-methyl-	$N_4SC_6H_6$	A
14575	196	9H-Purine-6-thiol, 9-ethyl-	$N_4SC_7H_8$	A
33184	202	Purine, 6-(methylthio)-9-(tetrahydro-2-pyranyl)-	$N_4OSC_{11}H_{14}$	A
42380	214	Purine, 2-amino-9-benzyl-6-(methylthio)-	$N_5SC_{13}H_{13}$	A
43405	62/268	Purine-6(1H)-thione, 2-amino-1-methyl-	$N_5SC_6H_7$	A
44579	216/634	9H-Purine, 6-(methylthio)-9-(tetrahydro-2-furyl)-	$N_4OSC_{10}H_{12}$	A
45152	628	9H-Purine, 6-iodo-9-(tetrahydro-2-furyl)-	$N_4OC_9H_9I$	A
46382	629	9H-Purine, 6-chloro-9-(tetrahydro-6-methoxy-2H-pyran-2-yl)-	$N_4ClO_2C_{11}H_{13}$	A
54258	158	Purine-6-thione, 1-methyl-	$N_4SC_6H_6$	A
54262	225/508	9H-Purine-9-ethanol, 6-mercapto-	$N_4OSC_7H_8$	A
55464	224	9H-Purine-9-ethanol, 6-(methylthio)-	$N_4OSC_8H_{10}$	A
55469	570	9H-Purine, 2-amino-6-(ethylthio)-9-β-D-ribofuranosyl-	$C_{12}H_{17}N_5O_4S$	A
56937	230	9H-Purine, 6-(methylthio)-9-tetrahydro-2-thienyl)-	$N_4S_2C_{10}H_{12}$	A
67616	637	9H-Purine-6-thiol, 9-(tetrahydro-5-methyl-2-furyl)-	$N_4SOC_{10}H_{12}$	A
70896	298	Adenine, 1-methyl-	$N_5C_6H_8$	A
95941	534	1H-Pyrazolo[3,4-d]pyrimidine-3-carbonitrile, 4-amino-	$N_6C_6H_4$	A
95965	295	1H-Imidazo[4,5-c]pyridine, 4-(methylthio)-1-β-D-ribofuranosyl-	$N_3O_4SC_{12}H_{15}$	A
99439	442	7-(β-D-ribofuranosyl)-pyrrolo[2,3-d]pyrimid-4-one	$N_8O_5C_{11}H_{13}$	A
99930	395	9H-Purine-9-methanol, 6-(octylthio)-	$N_4OSC_{14}H_{22}$	A
99943	398	9H-Purine-9-methanol, 6-[(4-hydroxy-2-butynyl)thio]-	$N_4O_2SC_{10}H_{10}$	A
100278	443	7-(β-D-ribofuranosyl)-pyrrolo-[2,3-d]-4-pyrimidine-4-thiol	$N_3SO_4C_{11}H_{13}$	A
100279	444	4-Dimethylamino-7-(β-D-ribofuranosyl)-pyrrolo[2,3-d]-pyrimidine	$N_4O_4C_{13}H_{18}$	B
101160	446	4-Methoxy-7-(β-D-ribofuranosyl) pyrrolo[2,3-d]-pyrimidine	$N_3O_5C_{12}H_{15}$	B
103791	447	4-Methylamino-7-(β-D-ribofuranosyl)-pyrrolo[2,3-d]-pyrimidine	$N_4O_4C_{12}H_{16}$	A
105826	449	4-Methylthio-7-(β-D-ribofuranosyl)-pyrrolo[2,3-d]pyrimidine	$N_3SO_4C_{12}H_{15}$	A
107519	450	7-(β-D-ribofuranosyl)-pyrrolo-[2,3-d]pyrimidine	$N_3O_4C_{11}H_{13}$	A
111361	346	Purine-6(1-H)-thione 9-(2-deoxy-α-D-erythropentopyranosyl)	$N_4O_3SC_{10}H_{12}$	B
111702	371	D-Dioxane-2-methanol,3,5-dihydroxy-6-[6-(methylthio)-9H-purin-9-yl]-	$N_4O_5C_{11}H_{14}S$	A
112521	361	1,2-diamino-inosine	$N_6O_5C_{10}H_{14}$	B

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
112522	362	Purine-6,8(1H,9H)-dione, 1,2-diamino-9-β-D-ribofuranosyl-	$N_6O_6C_{10}H_{14}$	B
112525	365	8-bromo-hypoxanthine	$N_4OBrC_5H_3$	B
113941	381	Adenine, 9-(2-deoxy-β-D-erythro-pentopyranosyl)-	$N_5O_3C_{10}H_{13}$	A
113943	383	7H-Pyrrolo [2,3-d]pyrimidine-5-carboxamide-4-amino-6-bromo-7-β-D-ribofuranosyl-	$N_5O_5BrC_{12}H_{14}$	A
113948	388	Inosine, 8-mercapto	$N_4O_5SC_{10}H_{12}$	A
113950	390	Inosine, 8-methoxy	$N_4O_6C_{11}H_{14}$	A
113951	391	7-H-Pyrrolo-[2,3-d]pyrimidine-5-carbonitrile-4-amino-6-bromo-7-β-D-ribofuranosyl	$N_5O_4BrC_{12}H_{12}$	A
114335	402	9H-Purine-9-methanol,6-(hexylthio)-	$N_4OSC_{12}H_{18}$	A
114336	403	Carbamic acid,methyl-[6-(propylthio)-9H-purin-9-yl]-methyl ester	$N_5O_2SC_{11}H_{15}$	A
114860	407	Adenine,9-β-D-allopyranosyl	$N_5O_5C_{11}H_{15}$	A
114862	409	Adenine,9-α-D-mannopyranosyl	$N_5O_5C_{11}H_{15}$	A
115603	413	9H-Purine-6-thiol,9-β-D arabinopyranosyl compd. with methanol (3:1)	$N_4O_4SC_{10}H_{12}\cdot\frac{1}{3}(CH_3OH)$	A
115604	414	9H-Purine-6-thiol,9-α-D-arabinopyranosyl-	$N_4O_4SC_{10}H_{12}$	A
115607	417	Guanine,9-β-D-xylofuranosyl-	$N_5O_5C_{10}H_{13}$	A
115963	523	9H-Purine-6-thiol,9-[D-gluco-1-(ethylthio)-2,3,4,5,6-pentahydroxyhexyl]-	$N_4O_5S_2C_{13}H_{20}$	A
116092	524	Guanine,7-β-D-ribofuranosyl-	$N_5O_5C_{10}H_{13}$	A
116093	525	Xanthine,7-β-D-ribofuranosyl-2-thio-	$N_4O_5SC_{10}H_{12}$	A
116097	529	7H-Pyrrolo[2,3-d]pyrimidine-5-carbonitrile,4-mercapto-7-β-D-ribofuranosyl-	$N_4O_4SC_{12}H_{12}$	A
116098	530	7H-Pyrrolo[2,3-d]pyrimidine-5-carbonitrile, 4-(methylthio)-7-β-D-ribofuranosyl-	$N_4O_4SC_{13}H_{14}$	A
116099	531	9H-Purine,2-amino-6,8-dichloro-9-β-D-ribofuranosyl	$N_5O_4Cl_2C_{10}H_{11}$	A
117838	430	7H-Pyrrolo[2,3-d]pyrimidine-5-carboxamide,4-amino-6-(methylthio)-7-β-D-ribofuranosyl-	$N_5O_5SC_{13}H_{17}$	A
117842	433	9H-Purine,2-amino-8-chloro-6-(methylamino)-9-β-D-ribofuranosyl-	$N_6O_4ClC_{11}H_{15}$	A
118425	453	Adenine,9-α-D-xylopyranosyl-	$N_5O_4C_{10}H_{13}$	A
118426	454	Adenine,9-β-D-arabinopyranosyl-	$N_5O_4C_{10}H_{13}$	A
119661	464	Adenine,9-β-D-allofuranosyl	$N_5O_5C_{11}H_{15}$	A
119663	466	Adenine,9-β-L-gulofuranosyl-	$N_5O_5C_{11}H_{15}$	A
119664	467	Adenine,9-α-D-mannofuranosyl-	$N_5O_5C_{11}H_{15}$	A

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
119845	469	Purine-6,8(1H,9H)-dione,2-amino-1-methyl-9-β-D-ribofuranosyl-6-thio-	$N_5O_5SC_{11}H_{15}$	A
119846	470	Purine-6,8(1H,9H)-dione,2-amino-1 methyl-9-β-D-ribofuranosyl-	$N_5O_6C_{11}H_{15}$	A
119848	472	Inosine-1,2 diamino-8-(benzyloxy)-	$N_6O_6C_{17}H_{20}$	A
119850	473	Inosine,1,2 diamino-2'-deoxy	$N_6O_4C_{10}H_{14}$	A
119851	474	Inosine, 1-amino	$N_5O_5C_{10}H_{13}$	A
121182	482	Adenine,9-(2-amino-2-deoxy-β-D-xylofuranosyl)-	$N_6O_3C_{10}H_{14}$	A
121927	491	Adenine, 3-(2-deoxy-α-D-erythropentofuranosyl)-	$N_5O_3C_{10}H_{13}$	A
122325	495	7H-Pyrazolo[4,3-d]pyrimidine-7-thione,1,6-dihydro-3-β-D-ribofuranosyl-	$N_4O_4SC_{10}H_{12}$	A
122326	496	Purine,6-(methylthio)-7-β-D-ribofuranosyl	$N_4O_4SC_{11}H_{14}$	A
123431	504	Adenine,9-β-L-fucopyranosyl-, hydrate (5:1)	$N_5O_4C_{11}H_{15} \cdot \frac{1}{5} H_2O$	A
123432	505	9H-Purine-6-thiol,2-amino-9-β-D-xylofuranosyl-, monohydrate	$N_5O_4SC_{10}H_{13} \cdot HOH$	A
123434	507	Formamidine,N'-(9-β-D-arabinofuranosyl-9H-purin-6-yl)N,N,-dimethyl-	$N_6O_4C_{13}H_{18}$	A
124148	511	7H-Pyrrolo[2,3-d]pyrimidine,4,5-dichloro-7-β-D-ribofuranosyl-	$N_3O_4Cl_2C_{11}H_{11}$	A
124149	512	7H-Pyrrolo[2,3-d]pyrimidine, 4-amino-5-chloro-7-β-D-ribofuranosyl-	$N_4O_4ClC_{11}H_{13}$	A
124150	513	4H-Pyrrolo[2,3-d]pyrimidine-4-thione,5-bromo-3,7-dihydro-	$N_3SBrC_6H_4$	A
124151	514	7H-Pyrrolo[2,3-d]pyrimidine,5-bromo-4-(hydroxyamino)-	$N_4OBrC_6H_5$	A
124154	515	7-H-Pyrrolo[2,3-d]pyrimidine, 4-amino-7-(3-deoxy-β-D-erythropentofuranosyl)-	$N_4O_3C_{11}H_{14}$	A
124160	516	7H-Pyrrolo[2,3-d]pyrimidine, 4-(methylamino)-5-nitro-	$N_5O_2C_7H_7$	A
124165	521	1H-Pyrazolo[4,3-d]pyrimidine, 7-(hydroxyamino)-3-β-D-ribofuranosyl-, monohydrochloride	$N_5O_5C_{10}H_{13} \cdot HCl$	A
124166	522	1H-Pyrazolo[4,3-d]pyrimidine, 7-(methylthio)-3-β-D-ribofuranosyl-	$N_4O_4SC_{11}H_{14}$	A
125626	546	Adenine,9-(2,3-anhydro-5-azido-5-deoxy-β-D-lyxofuranosyl)-	$N_8O_2C_{10}H_{10}$	A
125627	547	9H-Purine,2-amino-6-chloro-9-β-D-xylofuranosyl-	$N_5O_4ClC_{10}H_{12}$	A
125640	820	7H-imidazolo[4,5d]-y-triazine, 4-amino-7-β-D-ribofuranosyl	$N_6O_4C_9H_{12}$	A
125685	573	Purine-8-methanol,2,6 diamino-	$N_6OC_6H_8$	A

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
126876	618	Adenine, N-hydroxy-9-β-D-arabinofuranosyl	$N_5O_5C_{10}H_{13}$	A
127520	578	Purine-6,8(1H,9H)-dione,1,2-diamino-7-methyl-9-β-D-ribofuranosyl-	$N_6O_6C_{11}H_{16}$	A
127972	600	Adenine,9-[3-deoxy-3-(2-hydroxyethyl)-β-D-ribofuranosyl]-	$N_5O_4C_{12}H_{17}$	A
127973	601	Adenine,9-(3-deoxy-3-methyl-β-D-allofuranosyl)-	$N_5O_4C_{12}H_{17}$	A
127976	604	Adenine,9-(5-amino-2,3-anhydro-5-deoxy-β-D-lyxofuranosyl)-	$N_6O_2C_{10}H_{12}$	A
128664	607	7H-pyrrolo[2,3-d]pyrimidine-5-carboximidic acid, 4-amino-7-β-D-ribofuranosyl-, ethylester, monohydrochloride	$N_5O_5C_{14}H_{19} \cdot HCl$	A
128668	610	1H-pyrazolo[4,3-d]pyrimidine, 7-hydrazino-3-β-D-ribofuranosyl-	$N_6O_4C_{10}H_{14}$	A
128669	611	1H-pyrazolo[4,3-d]pyrimidine, 7-amino-3-β-D-ribofuranosyl-, 6-oxide	$N_5O_5C_{10}H_{13}$	A
128674	614	Adenine,9-(2,3-dideoxy-D-erythro-hex-2-enopyranosyl)-	$N_5O_3C_{11}H_{13}$	A
128889	615	Adenine, 3'-deoxy-3'-(methyl amino)-	$N_6O_3C_{11}H_{16}$	A
129804	619	9H-purine,2-amino-6-chloro-9-β-D-xylofuranosyl-, 2'-methanesulfonate, compd. with ethyl-ether (4:3)	$N_5O_6SClC_{11}H_{14} \cdot \frac{3}{4}(OC_4H_{10})$	A
130263	620	9H-purine,2,6-bis(hydroxyamino)-9-β-D-ribofuranosyl-	$N_6O_6C_{14}H_{14}$	A
130280	622	7H-v-triazolo[4,5d]pyrimidin-7-one, 5-amino-3-(2-deoxy-D-erythro-pentofuranosyl)-3,6-dihydro-, stereoisomers	$N_6O_4C_9H_{12}$	A
130281	623	Adenine, 8-(β-D-ribofuranosylthio)-	$N_5O_4SC_{10}H_{13}$	A
130283	624	7H-v-triazolo[4,5-d]pyrimidin-7-one, 5-amino-3,6-dihydro-3-β-D-ribofuranosyl-	$N_6O_5C_9H_{12}$	A
131605	650	9H-Purine, 9-(2-amino-2-deoxy-β-D-ribofuranosyl)-6-(methylthio)-	$N_5O_3SC_{11}H_{15}$	A
131663	657	7H-Pyrrolo[2,3-d]pyrimidine-5-carboxamidine, 4-amino-7-β-D-ribofuranosyl, dihydrochloride	$N_6O_4C_{12}H_{16} \cdot 2\ HCl$	A
132099	662	Adenosine, 3'-deoxy-N,N-dimethyl-3'-(methylamino)-	$N_6O_3C_{13}H_{20}$	A
132292	664	2(5H)-furanone, 5-(6-amino-9H-purin-9-yl)-4-ethyl-3-methyl-	$N_5O_2C_{12}H_{13}$	A
132355	665	Inosine,3'-deoxy-3'-(methylamino)-	$N_5O_4C_{11}H_{15}$	A
133114	675	Adenosine,5'-sulfamate, monohydrate	$N_6O_6SC_{10}H_{14} \cdot H_2O$	A
133117	677	Adenine, 8-bromo-3-β-D-ribofuranosyl-	$N_5O_4BrC_{10}H_{12}$	A
133119	679	3H-v-triazolo[4,5-d]pyrimidine, 7-(dimethylamino)-3-β-D-ribofuranosyl-	$N_6O_4C_{11}H_{16}$	A

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
133418	682	9H-purine-6-thiol,2-amino-9-β-D-arabinofuranosyl-, hydrate (4:1)	$N_5O_4SC_{10}H_{13}\cdot\frac{1}{4}$ HOH	A
133681	685	Ammonium, trimethyl(3-β-D-ribofuranosyl-1H-pyrazolo-[4,3-d]pyrimidin-7-yl)-, chloride	$N_5O_4C_{13}H_{20}\cdot$ Cl $N_5O_4ClC_{13}H_2O$	A
134680	692	9H-Purine-6-(1H)-thione, 9-[3-deoxy-3-(methylamino)-β-D-ribofuranosyl]-	$N_5O_3SC_{11}H_{15}$	A
134681	693	9H-Purine-6-thiol,2-amino-9(2,3-anhydro-β-D-lyxofuranosyl)-, hydrate (3:2)	$N_5O_3SC_{10}H_{11}\cdot\frac{2}{3}$ H_2O	A
134682	694	9H-purine-6-thiol,2-amino-9-β-D-xylofuranosyl-, 2'-methane sulfonate	$N_5O_6S_2C_{11}H_{15}$	A
135673	699	3H-furo[3,4-d]-1,3,2-oxazaphosphole-4-methanol, 2-[bis-(2-chloroethyl)amino]-6-[6-(dimethylamino)-9H-purin-9-yl]-3a,4,6,6a-tetrahydro-,2-oxide	$N_7O_4PCl_2C_{16}H_{24}$	A
136022	702	Adenosine,3'-deoxy-3'-(dimethylamino)-N,N-dimethyl	$N_6O_3C_{14}H_{22}$	A
136512	705	1H-imidazo[4,5-b]pyrazine,5,6-dimethyl-1-β-D-ribofuranosyl-	$N_4O_4C_{12}H_{16}$	A
144667	845	Adenine,9-(3-aminopropyl)-	$N_6C_{18}H_{12}$	A
144696	807	Ribofuranuronic acid, 1-(6-amino-9H-purin-9-yl)-1-deoxy-, methyl ester, hemihydrate, β-D-	$N_5O_5C_{11}H_{13}\cdot\frac{1}{2}$ HOH	A
144697	806	Ribofuranouronamide,1-(6-amino-9H-purin-9-yl)-1-deoxy-, monohydrate,β-D-	$N_6O_4C_{10}H_{12}\cdot$ HOH	A
144743	846	Threonine,N-(purin-6-ylcarbamoyl)-, L-	$N_6O_4C_{10}H_{12}$	A
145067	804	Adenine, N-(cyclopropylmethyl)-	$N_5C_9H_{11}$	A
145068	805	Adenine, 3-allyl-	$N_5C_8H_9$	A
145071	848	2-Butanol,3-methyl-1-(purin-6-ylamino)-	$N_5OC_{10}H_{15}$	A
145074	851	Adenine, N-isopentyl	$N_5C_{10}H_{15}$	A
145077	815	Adenine, 9-(3-chloropropyl)-	$N_5ClC_8H_{10}$	A
145173	817	Adenine, 9-(5-amino-5-deoxy-β-D-arabinofuranosyl)-	$N_6O_3C_{10}H_{14}$	A
145386	816	7H-pyrrolo[2,3-d]pyrimidine-5-carboxamid-oxime, 7-β-D-ribofuranosyl-	$N_5O_5C_{12}H_{15}$	A
145388	812	Formamidine,N'-(5-cyano-7-β-D-ribofuranosyl-7H-pyrrolo-[2,3-d]pyrimidin-4-yl)-N,N-dimethyl-, 3-oxide	$N_6O_5C_{15}H_{18}$	A
145389	819	7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide, 4-hydrazino-7-β-D-ribofuranosyl	$N_6O_5C_{12}H_{16}$	A

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
145391	803	Benzimidazole,2,5,6-trichloro-1-β-D-ribofuranosyl-	$N_2O_4Cl_3C_{12}H_{11}$	A
145393	818	1H-pyrazolo[4,3-d]pyrimidine, 7-amino-1-methyl-3-β-D-ribofuranosyl-	$N_5O_4C_{11}H_{15}$	A
145394	811	D-erithro-hex-1-enopyranose,3-(6-amino-9H-purin-9-yl)-1,2,3-trideoxy-	$N_5O_3C_{11}H_{13}$	A
407185+	549	9H-Purine,9-β-D-arabinofuranosyl-6-chloro	$N_4O_4ClC_{10}H_{11}$	A
407371+	550	Adenine,9-β-D-arabinofuranosyl-N,N-dimethyl-	$N_5O_4C_{12}H_{17}$	A
407734+	551	9H-Purine,9-β-D-arabinofuranosyl-	$N_4O_4C_{10}H_{12}$	A
—	54	Adenine 1-oxide	$N_5OC_5H_4$	C
—	563	9-(2-aminoethyl)adenine	$N_6C_7H_{10}$	C
—	625	1H-pyrazolo(3,4-d)pyrimidine, 1(2-cyanoethyl)-4(N,N-bis(2-cyanoethyl)amino)	$N_4C_{14}H_{14}$	C
—	644	1H-pyrazolo(3,4-d)pyrimidine, 1-methyl-3-cyanomethyl-4-amino-	$N_6C_8H_8$	C
—	668	9-(carboxymethyl)adenine	$N_5O_2C_7H_7$	C
—	749	1H,5H-4-iminopyrazolo(3,4-d)-pyrimidine, 1-methyl-5-hydroxyethyl-	$N_5OC_8H_{11}$	C
—	750	1H,5H-4-iminopyrazolo(3,4-d)-pyrimidine, 1-methyl-5-(2-aminoethyl)	$N_6C_8H_{11}$	C
—	751	1H-pyrazolo(3,4-b)pyridine 3-methyl-5-nitro-	$N_4O_2C_7H_6$	C
—	753	1H-pyrazolo(3,4-b)pyridine, 1-hydroxyethyl-3-amino-	$N_4OC_8H_{10}$	C
—	772	Pyrazolo(1,5-a)pyrimidine, 3-carboxy-5-methyl-	$N_3O_2C_8H_7$	C
—	830	1-β-D-ribofuranosyl-3-aminoindazole	$N_3O_4C_{12}H_{15}$	C
—	831	1-β-D-ribofuranosyl-3-nitropyrazolo(3,4-d)pyridine	$N_4O_6C_{11}H_{10}$	C
—	832	1-β-D-ribofuranosyl-3-amino-1,2,4-triazole	$N_4O_4C_7H_{12}$	C
—	835	1-β-D-ribofuranosyl-4-nitro-1,2,3-triazole	$N_4O_6C_7H_{10}$	C
—	855	4'-thiocordycepin	$N_5O_3C_{10}SH_{13}$	D
—	876	Adenosine,N^6-benzyl	$N_5O_4C_{17}H_{19}$	E
—	877	N^6-(Δ^2-isopentenyl)-arabinosyl adenine	$N_5O_4C_{15}H_{21}$	E
—	878	N^6-Benzyl-arabinosyl adenine	$N_5O_4C_{17}H_{19}$	E
—	879	2'-deoxy-N^6-(Δ^2-isopentenyl)-adenosine	$N_5O_3C_{15}H_{21}$	E
—	880	N^6-benzyl-2'-deoxyadenosine	$N_5O_3C_{17}H_{19}$	E
—	881	3'-deoxyadenosine,N^6-benzyl-	$N_5O_3C_{17}H_{19}$	E

TABLE A. -- List of compounds tested -- Continued

NSC No.	CRU No.	Compound	Molecular formula	Source*
—	882	Adenosine, N^6-(3-methyl-2-butenyl)-2'-O-methyl-	$N_5O_4C_{16}H_{24}$	E
—	898	2'-O-(N-acetyl)-L-phenylalanyl-3')-deoxyadenosine	$N_6O_5C_{21}H_{24}$	E
—	R-4	6-chloropurine-2'-deoxyriboside	$N_4Cl_1O_3C_{10}H_{11}$	E
—	R-9	6-fluoro-9-(2-deoxy-β-D-erythro-pentofuranosyl)-purine	$N_4F_1O_3C_{10}H_{11}$	E
—	R-5	6-mercapto-9-(2-deoxy-β-D-erythro-pentofuranosyl)-purine	$N_4S_1O_3C_{10}H_{12}$	E
—	R-8	9-(2-deoxy-β-D-erythro-pento-furanosyl)-purine-6-yl-tri-methyl ammonium chloride	$N_5Cl_1O_3C_{13}H_{20}$	E
—	R-11	6-hydroxylaminopurine-2'-deoxy-riboside	$N_5O_4C_{10}H_{13}$	E
—	B-1	Miconazole nitrate	$C_{18}H_{14}Cl_4N_2O \cdot HNO_3$	F
—	R-7	6-methylthio-9-(2-deoxy-β-D-erythro-pentofuranosyl)-purine	$N_4S_1O_3C_{11}H_{14}$	E
—	R-6	6-(p-nitrobenzylthio)-9-(2-deoxy-β-D-erythro-pentofuranosyl)-purine	$N_5S_1O_5C_{17}H_{17}$	E

* The source of each compound is coded as follows:

Code	Source
A	Drug Research and Development National Cancer Institute Bethesda, Md.
B	Dr. R. K. Robins ICN Nucleic Acid Research Institute Irvine, Calif.
C	Dr. B. M. Lynch St. Francis Xavier University Antigonish, Nova Scotia, Canada
D	Dr. W. A. Szarek Queen's University Kingston, Ontario, Canada
E	Dr. M. J. Robins University of Alberta Edmonton, Alberta, Canada
F	Dr. H. van den Bossche Janssen Pharmaceutica Beerse, Belgium

TABLE 1. -- Inhibitors of hypoxanthine metabolism in Ehrlich ascites tumor cells

NSC No.	CRU No.	Total nucleotides H→IMP	Adenine nucleotide synthesis: IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	Guanine nucleotide synthesis: IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
			Percent Inhibition								
7841	65	6.91	1.70	-1.63	2.37	6.78	-16.0	-1.76	-0.20	17.8	44.5
14575	196	-2.57	-0.10	0.16	1.23	10.4	-1.98	-6.22	-2.98	10.9	25.1
33184	202	10.4	-6.32	-0.55	3.55	28.2	20.3	-0.71	4.42	-21.3	-36.7
42380	214	-4.05	-4.02	-0.75	0.19	-7.5	19.1	0.20	-2.41	-16.4	-45.9
43405	62/268	73.8	0.61	-1.55	0.50	-7.78	5.63	5.77	-2.47	8.42	19.6
44579	216/634	6.41	-6.35	-0.06	5.01	39.7	21.1	-0.57	-1.11	-10.3	-25.4
45152	628	80.7	-1.3	0.3	4.1	28.9	31.4	0.9	2.1	-16.6	-87.5
46382	629	78.2	-2.3	-0.4	-0.2	-9.0	0.5	-1.8	-0.2	1.9	19.7
54258	158	15.1	-1.86	-0.21	0.83	4.15	10.0	-5.32	0.63	6.66	22.5
54262	225/508	14.0	4.29	-2.83	3.34	6.14	-2.22	0.31	-0.85	8.45	23.6
55464	224	11.3	3.71	-3.35	2.88	-2.80	2.48	-5.20	-4.75	-1.45	-28.1
55469	570	12.2	-6.58	-1.02	3.44	24.1	22.7	3.19	0.05	-37.6	-114
56937	230	34.4	-1.47	2.05	13.5	69.6	7.09	2.74	3.29	-3.86	-0.86
67616	637	75.4	7.5	-0.1	2.8	25.9	-54.1	19.1	0.9	15.9	73.6
70896	298	11.3	6.58	-3.11	2.69	-2.19	-17.08	3.17	-4.47	8.67	15.2
95941	534	-8.01	-14.1	-0.35	6.69	36.7	74.2	-26.8	15.8	57.9	88.4
95965	295	-8.71	-3.93	-0.98	-1.65	-48.2	-0.93	4.47	-2.73	5.65	5.84
99439	442	44.7	4.50	-3.28	-0.48	-24.0	-21.4	0.48	-1.13	-5.62	-26.5
99930	395	57.2	24.4	8.20	27.9	86.9	1.13	1.75	7.43	16.8	54.5
99943	398	-0.94	-3.84	-0.04	1.72	17.5	41.3	2.52	0.85	4.03	16.9
100278	443	-8.95	-8.16	-2.04	8.22	24.5	0.44	-0.97	-3.20	3.80	2.32
100279	444	79.5	3.37	-0.07	1.94	19.2	39.0	9.02	3.00	3.00	20.1
101160	446	38.7	-5.74	0.21	2.79	28.3	44.3	-0.94	1.86	-3.80	-7.95
103791	447	69.0	7.21	-4.79	0.42	-26.8	16.5	0.72	-4.40	9.80	16.9
105826	449	55.1	6.95	-3.41	38.2	76.3	53.8	4.11	-0.39	0.32	-0.22
107519	450	96.1	54.0	5.67	-10.3	81.6	62.8	26.5	3.01	91.2	97.2
111361	346	9.22	0.72	-0.12	2.95	36.4	-5.14	0.11	1.18	1.91	18.3
111702	371	23.5	-10.8	-1.05	-1.03	-34.8	54.2	12.8	1.41	21.2	36.0
112521	361	35.6	-6.28	0.53	7.30	62.2	84.1	-0.09	1.17	32.6	78.1
112522	362	-9.71	1.19	0.01	3.95	44.6	-9.49	-0.11	1.48	2.99	24.7
112525	365	3.98	1.33	0.16	1.80	27.7	-10.7	-0.18	1.56	0.68	13.9
113941	381	4.85	2.38	0.40	3.49	44.1	-23.1	0.17	1.35	7.80	41.3
113943	383	-6.22	-3.19	0.14	2.18	31.3	36.8	0.41	1.85	10.6	49.9
113948	388	-7.33	-0.74	1.02	2.81	33.1	6.35	2.96	0.40	-5.19	-23.8

TABLE 1. -- Inhibitors of hypoxanthine metabolism in Ehrlich ascites tumor cells -- Continued

NSC No.	CRU No.	Total nucleotides H→IMP	Adenine nucleotide synthesis: IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	Guanine nucleotide synthesis: IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
			Percent Inhibition								
113950	390	-13.0	0.53	0.58	0.02	10.5	-2.89	0.58	1.60	4.92	32.8
113951	391	-1.76	-0.73	0.09	0.51	10.3	12.3	0.41	1.30	6.50	37.2
114335	402	48.0	3.01	1.50	10.5	63.1	39.7	9.14	3.54	-1.81	6.83
114336	403	58.6	5.37	5.93	19.5	86.3	0.18	0.29	7.74	18.1	69.7
114860	407	-0.51	0.50	0.47	3.97	38.3	2.48	1.02	-0.38	-5.99	-34.1
114862	409	-8.51	2.13	0.31	-1.72	-25.4	-17.1	0.06	-0.38	10.2	31.6
115603	413	-11.5	-0.16	0.63	-1.31	-12.7	6.19	0.56	1.60	-4.06	-10.9
115604	414	-16.8	0.16	-0.16	-1.77	-33.3	0.80	0.95	1.32	12.1	39.4
115607	417	20.4	0.10	-0.97	10.1	33.4	-0.83	1.68	-1.10	3.06	6.28
115963	523	8.57	4.58	0.33	-1.27	-13.9	-19.0	10.6	-10.6	9.85	0.94
116092	524	40.8	-1.16	1.11	-2.28	-17.4	11.9	39.0	-6.45	14.4	25.5
116093	525	2.08	-2.50	0.61	-0.78	-3.2	13.7	1.32	-12.4	2.65	-43.1
116097	529	16.0	-3.33	0.88	-2.91	-43.1	17.9	-15.7	-12.0	14.1	12.3
116098	530	67.3	3.16	1.83	3.64	48.2	-3.5	21.8	-3.37	26.3	52.1
116099	531	29.1	-5.71	1.10	4.71	48.0	31.8	2.50	-4.97	11.3	20.5
117838	430	15.9	-7.19	-3.18	8.47	21.7	28.5	0.20	-2.04	17.3	37.9
117842	433	3.82	1.77	1.47	-0.35	11.1	5.97	-0.44	2.88	-1.85	4.6
118425	453	12.8	-2.81	-0.32	-2.71	-41.8	19.9	-7.18	0.60	-12.4	-68.8
118426	454	23.4	-3.54	-0.01	-1.59	-18.7	30.3	-7.60	1.14	-6.09	-21.9
119661	464	-5.66	-3.15	-0.09	-3.21	-33.4	26.8	-12.3	-0.45	-17.4	-108
119663	466	2.49	-2.78	-2.44	1.55	-3.96	16.4	-0.10	-1.15	-11.1	-56.9
119664	467	-2.50	-1.46	-0.07	1.45	9.43	15.3	-3.33	1.20	-9.36	-34.0
119845	469	8.63	1.28	-0.31	2.66	10.0	9.35	1.06	2.27	-0.19	6.54
119846	470	6.84	1.53	3.32	12.6	47.8	-1.18	1.40	3.08	11.9	36.1
119848	472	-24.4	-0.27	-0.18	-1.37	-25.1	8.86	0.84	1.98	-5.31	-15.2
119850	473	-15.4	-3.48	-0.24	0.41	0.38	35.3	1.84	2.41	8.77	34.2
119851	474	24.3	4.80	-3.11	1.01	-25.0	-0.71	-5.14	-2.55	6.99	15.3
121182	482	16.1	6.51	0.55	8.54	52.3	-12.8	-1.63	-2.72	2.39	-1.17
121927	491	5.38	1.27	-0.70	1.20	0.54	-6.23	-5.50	-0.19	1.75	5.13
122325	495	33.7	3.31	1.22	10.7	59.6	-2.28	0.28	-1.58	6.82	19.9
122326	496	4.22	0.16	0.47	1.62	12.7	9.22	-19.4	-1.37	5.79	13.8
123431	504	23.1	4.64	4.15	4.00	49.1	-8.24	-0.86	4.23	-0.35	15.1

TABLE 1. -- Inhibitors of hypoxanthine metabolism in Ehrlich ascites tumor cells -- Continued

NSC No.	CRU No.	Total nucleotides H→IMP	Adenine nucleotide synthesis: IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	Guanine nucleotide synthesis: IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
			Percent Inhibition								
123432	505	-9.93	-2.64	-0.48	1.10	3.75	14.5	-10.6	0.22	-9.00	-37.8
123434	507	2.31	0.51	1.05	1.73	16.6	22.7	-13.5	-0.88	-11.3	-59.9
124148	511	59.1	-3.27	6.53	23.1	81.2	35.6	-2.47	-0.76	20.6	52.5
124149	512	50.5	-5.23	0.26	1.28	15.9	22.4	4.46	-0.15	11.2	33.3
124150	513	-12.6	9.08	2.83	4.77	48.0	-17.8	7.28	8.31	9.67	47.7
124151	514	12.8	-0.22	0.10	-7.92	-65.5	1.39	-0.29	-4.96	4.28	-1.61
124154	515	91.3	45.6	1.26	0.93	23.2	-48.6	26.4	2.72	2.37	17.5
124160	516	5.53	-3.36	-1.58	-2.29	-25.7	9.96	0.18	-0.91	-1.46	-8.5
124165	521	1.28	-2.38	2.02	0.96	16.0	23.4	-12.0	0.83	-9.09	-34.7
124166	522	48.3	1.31	0.49	0.29	13.0	-4.51	18.9	-6.32	1.56	-18.3
125626	546	-5.28	-5.34	-1.59	-5.09	-115	15.8	-12.1	-5.20	2.90	-9.6
125627	547	-11.9	-3.43	-1.69	-6.23	-173	8.37	-10.6	-7.12	24.0	48.9
125640	820	95.2	22.1	-5.5	7.7	34.6	-88.1	-12.5	5.0	10.9	58.7
125685	573	-7.11	-6.50	-0.09	-2.31	-42.2	14.4	-1.13	-2.78	-14.0	-40.1
126876	618	-5.4	-13.6	-0.2	0.6	-12.3	49.2	1.0	1.4	-24.6	-230.9
127520	578	-12.0	-0.18	0.44	-0.44	-3.08	2.74	0.60	1.54	-2.32	-3.25
127972	600	4.4	-5.9	-0.4	-4.3	-83.9	22.1	1.0	0.5	-28.5	-452.5
127973	601	-0.1	-10.1	-1.2	-3.2	-77.5	33.7	0.7	0.7	-19.2	-139.9
127976	604	5.0	-6.9	0.4	-4.4	-65.8	28.2	4.0	1.7	-30.0	-444.2
128664	607	93.2	-12.6	5.7	-0.7	38.4	48.8	17.7	6.7	-14.2	-30.0
128668	610	58.5	-0.6	2.0	4.3	40.7	10.8	-0.2	1.5	-20.0	-138.1
128669	611	13.6	-3.6	1.0	-3.2	-30.1	24.0	1.5	1.3	-27.4	-335.1
128674	614	10.3	-6.1	6.1	10.9	64.4	28.9	0.8	2.5	-19.6	-115.3
128889	615	34.6	10.7	2.4	-2.1	0.1	-40.3	4.7	3.2	-17.6	-83.6
129804	619	43.3	5.3	4.4	14.9	68.5	23.3	3.4	4.9	-15.9	-53.8
130263	620	91.5	2.7	18.4	13.9	81.0	-0.5	3.9	17.9	-17.8	11.3
130280	622	1.6	8.7	2.2	-1.6	4.6	5.0	4.5	3.2	-24.6	-179.0
130281	623	8.3	1.2	-0.9	-4.3	-103.7	27.3	1.0	0.7	26.8	-337.7
130283	624	20.1	2.6	0.3	-1.1	-5.1	25.8	3.3	1.4	-27.4	-327.7
131605	650	-21.7	-4.90	-0.84	-2.90	-84.4	15.4	0.68	-2.92	-11.9	-34.7
131663	657	89.7	27.8	0.33	7.07	50.2	-16.3	47.9	3.70	-0.26	12.5
132099	662	9.4	-2.2	-0.2	0.7	5.8	0.0	2.1	-0.2	18.1	75.2

TABLE 1. -- Inhibitors of hypoxanthine metabolism in Ehrlich ascites tumor cells -- Continued

NSC No.	CRU No.	Total nucleotides H→IMP	Adenine nucleotide synthesis: IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	Guanine nucleotide synthesis: IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
			Percent Inhibition								
132292	664	94.8	36.6	-0.4	-0.8	-8.5	-142.8	10.5	-0.1	5.9	46.1
132355	665	4.3	3.1	0.3	1.8	21.5	-30.7	-0.5	-0.4	0.2	-2.6
133114	675	32.3	1.2	0.1	-1.1	-14.5	-23.1	4.1	0.4	5.4	45.9
133117	677	1.0	-4.1	0.2	-1.1	-12.3	-3.3	0.2	0.6	6.1	49.9
133119	679	-12.7	-3.4	0.2	-0.8	-7.0	-5.8	-0.5	0.4	3.7	36.9
133418	682	11.6	-3.1	0.8	5.8	47.7	8.9	-1.6	1.0	3.0	36.2
133681	685	12.9	-0.7	-0.9	-0.9	-17.6	3.1	-0.6	-2.5	-4.8	-41.2
134680	692	6.5	-0.5	-0.9	-0.6	-13.7	4.1	1.5	-4.0	-1.5	-28.4
134681	693	8.3	-0.5	-0.7	-0.8	-14.1	4.8	-1.2	-4.3	-3.7	-45.8
134682	694	4.6	5.6	-0.4	-5.1	-75.8	-54.0	-0.4	-4.3	-10.0	-115.8
135673	699	32.1	5.7	0.4	0.6	8.7	-35.3	-1.0	-3.9	-4.7	-51.2
136022	702	0.5	-4.9	-6.7	2.0	-38.1	18.3	-19.9	-1.2	5.1	24.2
136512	705	2.58	2.67	-3.37	-2.78	-125.5	-8.45	-6.42	-5.13	12.6	24.8
144667	845	87.9	15.1	1.48	1.06	22.9	-93.3	3.05	-5.77	-4.68	-74.1
144696	807	-6.7	-1.4	0.0	3.8	42.1	0.8	-3.9	-0.6	1.0	2.0
144697	806	-7.6	-3.2	-0.1	0.6	8.3	13.7	-2.4	0.1	-5.6	-42.7
144743	846	16.5	2.7	7.7	-2.8	24.1	-27.0	1.9	0.6	11.6	51.9
145067	804	70.5	34.5	40.0	62.0	98.4	-15.4	-3.6	38.5	48.4	92.5
145068	805	4.8	-1.6	0.0	3.1	36.9	4.9	-5.6	-0.6	7.7	30.8
145071	848	18.3	2.06	2.56	14.9	69.3	16.1	-2.73	-2.77	7.96	20.1
145074	851	74.8	-6.0	1.8	8.7	63.8	48.9	2.6	3.0	8.7	58.6
145077	815	-20.6	1.4	0.2	2.9	36.3	-25.7	2.5	1.0	22.4	63.4
145173	817	-8.3	3.9	0.2	2.9	36.4	-13.9	7.8	-0.6	3.0	12.6
145386	816	32.9	-1.5	0.4	1.1	20.6	-8.4	3.6	0.3	7.1	31.5
145388	812	-3.9	-2.5	0.1	2.8	34.0	10.1	1.0	2.3	2.2	21.2
145389	819	36.9	2.8	0.3	5.3	51.3	-30.6	5.0	0.4	11.0	42.5
145391	803	59.9	28.8	45.3	60.3	98.5	5.2	-3.3	44.3	45.1	92.9
145393	818	17.0	5.2	0.2	2.6	34.5	-29.3	8.7	-0.3	3.1	14.3
145394	811	-7.2	1.2	0.0	2.6	32.6	-6.2	4.0	0.6	3.6	20.4
407185+	549	12 6	-0.43	-0.81	-0.21	-6.2	-15.6	-1.26	-0.02	20.9	47.2
407371+	550	4.89	-1.16	-1.52	-2.69	-28.1	-14.3	-2.23	-3.96	19.1	39.1
407734+	551	-3.68	4.52	-0.79	-2.73	-22.7	-66.4	-1.70	-1.79	41.1	69.3
—	54	18.8	1.63	2.27	-0.13	16.2	-10.5	2.95	-0.20	-7.96	-39.2

TABLE 1. -- Inhibitors of hypoxanthine metabolism in Ehrlich ascites tumor cells -- Continued

NSC No.	CRU No.	Total nucleotides H→IMP	Adenine nucleotide synthesis: IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	Guanine nucleotide synthesis: IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
			Percent Inhibition								
—	563	50.8	4.93	2.75	-12.9	-94.7	-68.4	0.34	-5.20	10.6	17.7
—	625	4.6	3.7	0.2	-0.6	-0.3	21.3	3.6	1.7	-26.4	-275.5
—	644	7.9	3.2	0.0	1.6	17.3	-35.0	3.3	0.3	25.1	82.5
—	668	10.7	4.6	0.5	3.4	34.1	-22.6	0.9	0.1	1.2	15.7
—	749	-2.6	-2.5	-0.2	3.1	34.7	14.4	-3.6	-0.3	-1.0	-8.6
—	750	-5.9	-4.0	0.2	7.0	58.0	19.7	-4.4	0.2	-1.1	-5.3
—	751	29.9	-8.2	2.1	14.4	77.5	59.7	1.7	1.4	-3.7	-15.0
—	753	4.0	4.7	-0.2	-2.7	-29.4	-34.8	0.3	-3.0	2.2	-3.2
—	772	4.0	2.9	0.1	-4.3	-48.1	-20.7	0.0	-2.3	-2.2	-22.4
—	830	-4.8	-5.2	-5.2	-3.2	-81.9	31.3	-8.0	1.3	3.2	26.7
—	831	-8.5	-5.3	-10.1	0.1	-116.0	19.0	-24.9	0.6	3.3	23.8
—	832	-1.8	0.3	-7.7	-2.0	-103.9	-7.3	-12.7	-0.2	17.9	62.7
—	835	2.8	-3.1	-6.5	-1.0	-70.1	7.7	-13.1	-0.5	17.2	61.2
—	855	43.9	8.7	-10.5	-3.2	-249.8	-46.6	-21.4	-1.8	0.3	-12.7
—	876	70.4	-0.1	-0.6	6.4	50.0	19.6	0.0	-1.2	8.8	23.0
—	877	31.9	1.7	-0.2	0.0	-5.0	-20.9	0.6	1.4	-8.1	-28.1
—	878	67.0	3.7	0.6	7.6	59.0	-57.3	0.0	0.1	-8.9	-40.1
—	879	68.8	2.4	0.1	7.0	55.4	-36.8	0.0	0.8	3.9	14.9
—	880	78.3	1.2	2.4	9.4	59.8	7.1	0	-2.2	8.3	29.8
—	881	73.5	3.7	3.4	16.1	79.3	-50.5	0.0	4.0	-1.9	7.5
—	882	80.7	4.8	0.8	10.0	66.2	-58.3	0.0	-1.2	-11.6	-67.8
—	898	53.5	0.2	-0.3	4.3	41.0	-0.3	0.0	-1.2	-4.9	-25.4
—	R-4	71.8	-0.7	-2.0	0.5	-20.9	20.0	5.1	-1.7	-1.3	-22.5
—	R-9	90.5	21.6	2.3	8.3	56.9	-117.3	4.6	-2.2	2.6	3.8
—	R-5	60.3	-3.2	-0.1	2.3	21.7	39.2	5.7	17.6	9.6	68.5
—	R-8	42.8	4.7	0.7	1.7	34	-15.4	-0.1	-1.2	4.0	15.8
—	R-11	80.6	10.0	-1.5	-0.2	-25.0	-65.6	0.9	-2.5	-3.5	-57.3
—	B-1	42.4	2.5	-0.8	3.5	31.2	-20.2	-2.5	13.9	4.7	63.6
—	R-7	-0.2	-0.4	-1.2	9.8	52.3	6.4	-0.3	-0.9	10.2	39.6
—	R-6	6.9	-1.7	-1.4	1.3	-1.0	5.3	0.7	1.2	-0.5	4.1

TABLE 2.—Inhibitors of hypoxanthine phosphoribosyltransferase

NSC No.	CRU No.	% inhibition
107519	450	96.1
125640	820	95.2
132292	664	94.8
128664	607	93.2
130263	620	91.5
124154	515	91.3
—	R-9	90.5
131663	657	89.7
144667	845	87.9
45152	628	80.7
—	882	80.7
—	R-11	80.6
100279	444	79.5
—	880	78.3
46382	629	78.2
67616	637	75.4
145074	851	74.8
43405	62/268	73.8
—	881	73.5
—	R-4	71.8
145067	804	70.5
—	876	70.4
103791	447	69.0
—	879	68.8
116098	530	67.3
—	878	67.0
—	R-5	60.3
145391	803	59.9
124148	511	59.1
114336	403	58.6
128668	610	58.5
99930	395	57.2
105826	449	55.1
—	898	53.5
—	563	50.8
124149	512	50.5

TABLE 3.—Inhibitors of adenylosuccinate synthetase plus adenylosuccinate lyase

NSC No.	CRU No.	% inhibition
107519	450	54.0
124154	515	45.6
132292	664	36.6
145067	804	34.5
145391	803	28.8
131663	657	27.8
99930	395	24.4
125640	820	22.1
—	R-9	21.6

TABLE 4.—Inhibitors of the conversion of ADP to ATP

NSC No.	CRU No.	% inhibition
145067	804	62.0
145391	803	60.3
105826	449	38.2
99930	395	27.9
124148	511	23.1

TABLE 5.—Inhibitors of IMP dehydrogenase

NSC No.	CRU No.	% inhibition
112521	361	84.1
95941	534	74.2
107519	450	62.8
—	751	59.7
111702	371	54.2
105826	449	53.8
126876	618	49.2
145074	851	48.9
128664	607	48.8
101160	446	44.3
99943	398	41.3
114335	402	39.7
—	R-5	39.2
100279	444	39.0
113943	383	36.8
124148	511	35.6
119850	473	35.3
127973	601	33.7
116099	531	31.8
45152	628	31.4
—	830	31.3
118426	454	30.3

TABLE 6.—Inhibitors of GMP synthetase

NSC No.	CRU No.	% inhibition
131663	657	47.9
116092	524	39.0
107519	450	26.5
124154	515	26.4
116098	530	21.8

TABLE 7.—Inhibitors of the conversion of GDP to GTP

NSC No.	CRU No.	% inhibition
107519	450	91.2
95941	534	57.9
145067	804	48.4
145391	803	45.1
407734+	551	41.1
112521	361	32.6

TABLE 8.—Inhibitors of the ATP:ADP + AMP ratio

NSC No.	CRU No.	% inhibition
145391	803	98.5
145067	804	98.4
99930	395	86.9
114336	403	86.3
107519	450	81.6
124148	511	81.2
130263	620	81.0
—	881	79.3
—	751	77.5
105826	449	76.3
56937	230	69.6
145071	848	69.3
129804	619	68.5
—	882	66.3
128674	614	64.4
145074	851	63.8
114335	402	63.1
112521	361	62.2
—	880	59.8
122325	495	59.6
—	878	59.0
—	750	58.0
—	R-9	56.9
—	879	55.4
—	R-7	52.3
121182	482	52.3
145389	819	51.3
131663	657	50.2

TABLE 9.—Inhibitors of the GTP:GDP + GMP ratio

NSC No.	CRU No.	% inhibition
107519	450	97.2
145391	803	92.9
145067	804	92.5
95941	534	88.4
—	644	82.5
112521	361	78.1
132099	662	75.2
67616	637	73.6
114336	403	69.7
407734+	551	69.3
—	R-5	68.5
—	B-1	63.6
145077	815	63.4
—	832	62.7
—	835	61.2
125640	820	58.7
145074	851	58.6
99930	395	54.5
124148	511	52.5
116098	530	52.1
144743	846	51.9

TABLE 10.—Inhibitors of 2 or more enzymes

		Enzyme or process inhibited (from tables 2–9)									
		Total nucleotides	Adenine nucleotide synthesis				Guanine nucleotide synthesis				
NSC No.	CRU No.	H → IMP	IMP → AMP	AMP → ADP	ADP → ATP	ATP: AMP + ADP	IMP → XMP	XMP → GMP	GMP → GDP	GDP → GTP	GTP: GMP + GDP
45152	628	+					+				
67616	637	+									+
95941	534						+			+	+
99930	395	+	+		+	+					+
100279	444	+					+				
105826	449	+			+	+	+				
107519	450	+	+			+	+	+		+	+
112521	361					+	+			+	+
114335	402					+	+				
114336	403	+				+					+
116098	530	+						+			+
124148	511	+			+	+	+				+
124154	515	+	+					+			
125640	820	+	+								+
128664	607	+					+				
130263	620	+				+					
131663	657	+	+			+		+			
132292	664	+	+								
145067	804	+	+	+	+	+			+	+	+
145074	851	+				+	+				+
145391	803	+	+	+	+	+			+	+	+
407734+	551									+	+
—	751					+	+				
—	878	+				+					
—	879	+				+					
—	880	+				+					
—	881	+				+					
—	882	+				+					
—	R-9	+	+			+					
—	R-5	+					+				+

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Preclinical Toxicologic Evaluation of Dianhydrogalactitol in Dogs and Monkeys[1,2]

Roger M. Folk,* Kenneth L. Pavkov, and Daryl C. Thake[3]

SUMMARY

Thirty dogs in four different treatment schedules and 14 monkeys in a single multiple-treatment schedule were used to evaluate the toxicity of dianhydrogalactitol. Highest nontoxic, low toxic, high toxic, and lethal doses were established in single injection doses and five daily injections in dogs, and in five daily injections in monkeys. Dose ranges of 20–320 mg/m² (single injection) and 5–80 mg/m² (five daily injections) in dogs, and 3–96 mg/m² (five daily injections) in monkeys were established. The monkeys were more sensitive than dogs to the low toxic dose and more tolerant to the high toxic dose in the repeated daily injections. The dose-response curves for the dogs and monkeys had similar slopes and inflection points. Because of the steep slope between the lethal dose and the highest nontoxic dose in both species, caution should be used in the initial clinical trials.

[Cancer Treat Rep 60:1585–1589, 1976]

Dianhydrogalactitol is a diepoxy derivative of dibromodulcitol in alkaline environment. The compound demonstrates marked tumor inhibition in vitro against the NK/Ly ascites tumor, S180 sarcoma, Harding-Passey melanoma, Ehrlich ascites tumor, and the murine lymphocytic L1210 leukemia (1–3). The compound was selected for preclinical toxicity studies in dogs and monkeys because of its stability, its water solubility, an increase in lifespan (ILS) of 274% in the Ehrlich ascites tumor system, and an impressive biologic efficacy against the L1210 tumor with an ILS of > 400%.

MATERIALS AND METHODS

The dogs (purebred beagles, weight range 8–12.5 kg) and monkeys (rhesus, weight range 2.5–4.8 kg) were housed in individual steel cages. The dogs were fed Purina dog meal daily and were provided water ad libitum. Monkeys were fed Purina monkey chow twice daily and were provided water ad libitum.

Prior to being placed on study, fecal examinations for parasites were performed in dogs and monkeys and infested animals were appropriately treated. The monkeys were held in isolation for a minimum of 6 weeks and were tuberculin negative three consecutive times before they were released for treatment. At least two complete hematologic and blood chemical determinations and one urinalysis were performed on each dog and monkey before treatment. Body weight measurement and body temperature were obtained at least once a week during the study. Blood was obtained from treated animals and controls twice a week during the treatment period and once a week during the observation (posttreatment) period. The treatment schedules used in this evaluation are presented in table 1.

Both dogs and monkeys were treated in pairs, one male and one female at each dosage level. Complete necropsies were performed on all animals and > 40 tissues/animal were submitted to gross and histopathologic evaluation. Tissues were fixed with 10% neutral buffered formalin solution for preparation of paraffin sections. Eyes were fixed in 3% glutaraldehyde solution buffered at pH 7.2–7.25. Sections were routinely stained with hematoxylin and eosin and special stains were employed as required to provide a definitive evaluation.

[1]Supported by contract NIH-71-2262 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Dianhydrogalactitol: NSC-132313; CAS reg. No. 23261-20-3; galactitol, 1,2:5,6-dianhydro-.

[3]Battelle, Columbus Laboratories, Ohio.

*Reprint requests to: Dr. Roger M. Folk, Battelle, Columbus Laboratories, 505 King Ave, Columbus, Ohio 43201.

TABLE 1.—Treatment schedules and dose ranges used for toxicologic evaluation of dianhydrogalactitol in dogs and monkeys

Species	Study	Treatment schedule	Dose range (mg/m^2)
Dog	I	Single injection	20–320
	II	Daily for 5 days	5–80
	IV	Daily on Days 1–5, 15–19, 29–33, with rest periods on Days 6–14, 20–28	20–40
	V	Once per wk for 6 wks	20–40
Monkey	III	Daily for 5 days	3–96

The chemical identity of dianhydrogalactitol was verified by infrared spectroscopy and melting point determination. The drug was stored at 4°C. Stability of the drug in a 5% aqueous solution was determined previously at the Stanford Research Institute and the drug was found to be completely stable for a 24-hour period at room temperature. The solubility in 0.85% NaCl was determined to be > 160 mg/ml, four times the starting dose formulation. Appropriate concentrations ranging from 5 to 160 mg/ml were prepared in sterile 0.85% saline solution immediately before iv treatment.

The studies were performed and levels of toxicity[1] (tables 1 and 2) were determined according to the protocols of the Laboratory of Toxicology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (4).

RESULTS

There were no indications of sex-specific toxicity. All drug-related changes were reversed by the end of the 45-day observation period with the exception of extramedullary hematopoiesis, a residual effect of bone marrow depression.

[1]Defined levels of toxicity employed by the Laboratory of Toxicology are as follows:

Highest nontoxic dose (HNTD): the highest dose at which no hematologic, chemical, clinical, or pathologic drug-induced alterations occur; doubling this dose produces the aforementioned alterations.

Toxic dose low (TDL): the lowest dose to produce drug-induced pathologic alterations in hematologic, chemical, clinical, or morphologic parameters; doubling this dose produces no lethality.

Toxic dose high (TDH): the dose which produces drug-induced pathologic alterations in hematologic, chemical, clinical, or morphologic parameters; doubling this dose produces lethality.

Lethal dose (LD): the lowest dose to produce drug-induced death in any animal during the treatment or observation period.

TABLE 2.—Defined toxicity levels of dianhydrogalactitol in dogs and monkeys

Species	Study	Toxicity	Dose	
			mg/kg	mg/m^2
Dog	I	LD	16.0	320
		TDH	8.0	160
		TDL	2.0	40
		HNTD	1.0	20
Dog	II	LD	4.0	80
		TDH	2.0	40
		TDL	0.5	10
		HNTD	0.25	5
Monkey	III	LD	8.0	96
		TDH	4.0	48
		TDL	0.5	6
		HNTD	0.25	3

Study I

Five pairs of dogs were inoculated iv with single doses of dianhydrogalactitol, one pair at each of the following levels: 16, 8, 4, 2, or 1 mg/kg (320, 160, 80, 40, or 20 mg/m^2) (5). Of the two dogs treated with 16 mg/kg (320 mg/m^2), one died on Day 5 (Day 1 defined as the first day of treatment); the other was moribund and euthanized on Day 5. Anorexia, adipsia, hemorrhagic diarrhea, lethargy, and weight loss were the clinical signs of toxicity in animals treated with 16 or 8 mg/kg (320 or 160 mg/m^2). Less severe manifestations of the same clinical signs were observed at the 4-mg/kg (80 mg/m^2) level while only transient signs of anorexia and adipsia occurred in one animal treated with 2 mg/kg.

A slight to moderate decrease in the hematocrit, hemoglobin, and rbc values was observed at the highest dosage levels. An exception occurred in one animal treated with 16 mg/kg (320 mg/m^2) where these values were increased on Day 5 just prior to death, presumably due to dehydration and hemoconcentration. A marked decrease in the wbc count was also observed at the highest dosage levels. Alkaline phosphatase (AP) was increased in all animals treated with 16 or 8 mg/kg (320 or 160 mg/m^2). Serum glutamic oxaloacetic transaminase (SGOT) was elevated in one animal given 16 mg/kg. Sulfobromophthalein (BSP) retention and lactic dehydrogenase (LDH) levels were elevated in one animal at the 8-mg/kg (160 mg/m^2) level that was euthanized on Day 46. Lymphopenia was present in one animal given 8 mg/kg (160 mg/m^2). At the 4-mg/kg (80 mg/m^2) level, the total wbc count was decreased while AP and total bilirubin levels were increased.

Lymphoid depletion and bone marrow depression occurred in dogs treated with doses of 4 mg/kg (80 mg/m^2) or higher. Lymphoid depletion occurred in

virtually all lymphoid organs at the two highest dosage levels. Reticuloendothelial (RE) cell hyperplasia was apparent at these levels and extramedullary hematopoiesis was prominent in dogs necropsied after the 45-day observation period. Severe mucosal lesions were evident in the small intestine and colon of dogs given 16 or 8 mg/kg (320 or 160 mg/m^2). Lesions in the small intestine were characterized by markedly shortened villi, flattened or cuboidal villus epithelial cells, degeneration and necrosis of villus tips, dilated crypts, degenerate crypt epithelium, and decreased numbers of mitotic figures in crypt epithelial cells. Changes in the colon were similar considering the normal absence of villi in this portion of the intestine. Intestinal changes were not observed at dosage levels < 8 mg/kg (160 mg/m^2). In dogs treated with 4 mg/kg (80 mg/m^2), lymphoid depletion was less severe and primarily involved lymph nodes. RE cell hyperplasia and extramedullary hematopoiesis were observed at the 4- and 2-mg/kg (80 and 40 mg/m^2) levels.

Study II

Six pairs of dogs were used in this study. One pair was used as a control and five pairs were treated once daily for 5 consecutive days, one pair at each of the following levels: 4, 2, 1, 0.5, or 0.25 mg/kg (80, 40, 20, 10, or 5 mg/m^2). Dogs treated with 4.0 mg/kg (80 mg/m^2) died or were euthanized in a moribund state on Days 7 and 8 respectively. Anorexia, adipsia, weakness, lethargy, and weight loss were observed at the highest level and similar but less prominent signs were noted at the 2-mg/kg (40 mg/m^2) level.

Changes in hematologic and serum chemistry values were generally similar to those of study I. Changes at the two highest dosage levels, 4 and 2 mg/kg (80 and 40 mg/m^2), included depression of the total wbc count with marked neutropenia, slight decrease in rbc, hemoglobin, and hematocrit values, and elevation of AP levels. At dosage levels < 2 mg/kg (40 mg/m^2) total wbc counts were decreased with neutropenia present to a mild degree at levels down to 0.5 mg/kg (10 mg/m^2).

Generalized lymphoid depletion, thymic atrophy, tonsillar necrosis, and severe bone marrow depression were observed in animals treated with 4 mg/kg (80 mg/m^2). Intestinal lesions were less severe than in animals treated at the high dosage levels of study I. Mild to moderate mucosal degeneration and villus atrophy were present at the 4-mg/kg (80 mg/m^2) level. Lymphoid depletion, primarily involving lymph nodes, was observed in animals given 2 mg/kg (40 mg/m^2). Mild bone marrow depression occurred at levels as low as 0.5 mg/kg (10 mg/m^2). Extramedullary hematopoiesis was present in all animals treated with ≥ 0.5 mg/kg (10 mg/m^2) and euthanized at 50 days.

Study III

Seven pairs of monkeys were used for this study. One pair was used as a control and six pairs were treated once daily for 5 consecutive days, one pair at each of the following levels: 8, 4, 2, 1, 0.5, or 0.25 mg/kg (96, 48, 24, 12, 6, or 3 mg/m^2). Death occurred in both monkeys treated with 8 mg/kg (96 mg/m^2) on Day 8; one died spontaneously and the other was euthanized in a moribund state. Severe weight loss, anorexia, adipsia, lethargy, and hemorrhagic diarrhea occurred in monkeys treated with 8 mg/kg (96 mg/m^2). The same symptoms occurred at the 4-mg/kg (48 mg/m^2) level with the exception of diarrhea. The only symptom observed at a lower dosage level was mild weight loss in one monkey given 2 mg/kg (24 mg/m^2).

There was a decrease in the total wbc count in monkeys treated at the highest dosage levels, and neutropenia occurred in one animal at each of the two highest dosage levels. A moderate lymphopenia was present in one animal at the 8-mg/kg (96 mg/m^2) level. Marginal decreases in hematocrit, hemoglobin, and rbc values occurred in several animals treated at higher dosage levels. The only change in hematologic or serum chemistry values at lower dosage levels was a decreased total wbc which was present in decreasing severity down to the 0.5-mg/kg (6 mg/m^2) level.

Tissue changes were, for the most part, confined to monkeys treated at the two highest dosage levels. Lesions observed were similar to those described previously for dogs in studies I and II. Lymphoid depletion and bone marrow depression were prominent at both doses. Degeneration and atrophy of the intestinal mucosa were prominent at the 8-mg/kg (96 mg/m^2) level, while only mild changes occurred at the 4-mg/kg (48 mg/m^2) level. Degeneration of the esophageal and gastric mucosa was also present in the animals treated with 8 mg/kg (96 mg/m^2), as was vacuolation and swelling of liver hepatocytes. The only lesion which occurred at doses < 4 mg/kg (48 mg/m^2) was fibrous adhesion around the saphenous vein at the site of drug injection.

Study IV

Two pairs of dogs were used in this study. The treatment schedule was five consecutive daily injections through three treatment periods with intervening 9-day rest periods. Dosage levels used were 2.0 and 1.0 mg/kg (40 and 20 mg/m^2).

Of the two animals treated with 2 mg/kg, one died

on Day 22 and the other was euthanized in a moribund state on Day 12. Weight loss occurred in all animals treated at both dosage levels. Anorexia, adipsia, hemorrhagic diarrhea, and lethargy were prominent in animals treated at the higher dose while only mild anorexia and adipsia were noted at the lower dosage level.

Neutropenia was observed in all animals at both dosage levels, but there was considerable fluctuation within a given animal with little correlation to treatment and rest periods. The total wbc count was consistently lowered in all animals. Hematocrit, hemoglobin, and rbc values, although slightly decreased from baseline levels, generally remained in the accepted normal range. Platelet counts were decreased in both animals at the higher dosage level. AP levels were increased in three of the four dogs and serum glutamic pyruvic transaminase (SGPT) was elevated in one animal at the 2-mg/kg (40 mg/m^2) level. Serum calcium levels were slightly lowered in both animals given 2 mg/kg (40 mg/m^2).

Lymphoid depletion and bone marrow depression were present in all animals. Pulmonary congestion with hemorrhage and edema were prominent in both animals at the higher dose. Necrosis of the ventral portion of the apical lobe of the lung was observed in one dog treated with 2 mg/kg (40 mg/m^2). Changes observed in the intestinal tract at the higher dosage levels in other studies were not present in this study.

Study V

Two pairs of animals were used in this study. The treatment schedule was six consecutive weekly injections at 2-mg/kg (40 mg/m^2) and 1-mg/kg (20 mg/m^2) levels. There was little difference in the toxic response of dogs treated at either dosage level. The doses used were not lethal and clinical signs included mild weight loss, anorexia, and adipsia. A mild to moderate decrease in the wbc count was observed in both dogs given 2 mg/kg (40 mg/m^2). Moderate neutropenia and a marginal decrease in hemoglobin, hematocrit, and rbc values were observed in one animal from each dosage level. Mild bone marrow depression occurred at 2 mg/kg (40 mg/m^2).

DISCUSSION

The toxicity of dianhydrogalactitol was limited to tissues possessing large populations of rapidly dividing cells, ie, bone marrow, gastrointestinal mucosa, and lymphoid tissue.

Qualitative manifestations of toxicity were similar in all treatment schedules used in dogs. Clinical signs of weight loss, weakness, lethargy, anorexia, or adipsia were manifest to varying degrees in dogs depending on the dosage level and treatment regimen. Hemorrhagic diarrhea was observed in dogs in which there were moderate to severe intestinal lesions.

Bone marrow depression and lymphoid depletion were consistent in all treatment regimens. This could generally be correlated with depression of the total wbc count. Neutropenia was prominent in most dogs at higher dosage levels. In some animals, relative percentages of circulating leukocytes changed very little, while in three dogs given $\geq$ 8.0 mg/kg (160 mg/m^2), a relative and absolute lymphopenia was observed. Hematocrit, hemoglobin, and rbc values were decreased from baseline levels in animals treated on all treatment schedules. In most instances, these changes were slight to moderate, often staying within or near the accepted normal range. Platelet levels often fluctuated in parallel with other elements of the circulating blood; however, their values usually remained within the normal range. Platelet values dropped significantly below normal levels in two dogs that were treated on multiple consecutive-dose study IV treatment regimens. Hemorrhages were evident at necropsy in these two dogs.

AP values were elevated in most dogs treated at higher dosage levels on all treatment schedules except that which consisted of six weekly inoculations. BSP retention, LDH, SGOT, and SGPT values were sporadically elevated in several dogs but never were all increased in the same dog. The microscopic lesions which may correlate with these changes were present in the liver and consisted of hepatocyte vacuolation and centrilobular congestion.

Other serum chemistry changes included a mild decrease in serum calcium values and elevation of serum cholesterol levels in dogs treated at higher dosage levels on most treatment schedules. Extramedullary hematopoiesis was evident in dogs given treatment which induced bone marrow depression.

Intestinal lesions were prominent in animals that received single doses of 16 or 8 mg/kg (320 or 160 mg/m^2) in study I. Changes were less severe in dogs that received multiple-dose treatment regimens in studies II, IV, and V; the highest dosage level used in these schedules was 4 mg/kg (80 mg/m^2), suggesting a relatively high threshold of susceptibility for intestinal crypt epithelium. Intestinal lesions were characterized by degeneration of crypt and villus epithelium, decreased mitosis in crypt epithelial cells, and villus atrophy in the small intestine. Changes in the colon were qualitatively and quantitatively similar considering the absence of villi in this portion of the intestine.

The response in monkeys was similar to that described for dogs. Lymphoid depletion and bone marrow suppression were the most sensitive indicators of toxicity. Intestinal lesions occurred in animals treated at the highest dosage levels. Degenerative changes were also present in esophageal and gastric mucosa in monkeys. Serum chemistry values remained within the normal range while changes in hematologic parameters were confined to depression of the total wbc count and marginal decreases in hematocrit, hemoglobin, and rbc values.

Monkeys were more sensitive than dogs to dianhydrogalactitol at the TDL; the TDL in monkeys was 6 mg/m^2 for 5 consecutive days compared to 10 mg/m^2 in dogs for 5 consecutive days. However, monkeys were more tolerant than dogs at the TDH of 48 mg/m^2 compared to 40 mg/m^2 and had a higher established LD of 96 mg/m^2 compared to 80 mg/m^2. Death occurred on Day 7 in dogs and on Day 8 in monkeys.

The dose-response curves for the dogs and monkeys had similar slopes and inflection points. Because of the steep slope between the LD and the HNTD in both species, caution should be used in the initial clinical trials.

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ANNOUNCEMENTS

The Continuing Medical Education Institute Course on "The Changing Seasons of Life: The Physician's Role"

S. S. Rotterdam, New York to Nassau and Bermuda, April 23–30, 1977

The Continuing Medical Education Institute course on "The Changing Seasons of Life: The Physician's Role" to be held aboard the S. S. Rotterdam (New York to Nassau and Bermuda) April 23–30, 1977, is being sponsored by Sea and Shore Seminars, Washington, DC, the Charles R. Drew Postgraduate Medical School, Office of Continuing Education, and the University of Cincinnati, College of Medicine, Office of Continuing Medical Education. The registration fee is $175.00 (after February 23, 1977, the fee will be $200.00).

Medical problems of interest to both generalists and specialists will be presented in a continuum, prenatal through geriatric years. The course is acceptable for 25 credit hours in Category I for the Physician's Recognition Award of the American Medical Association. Additional professional areas for which accreditation will be available will be sent on request.

For further information contact: Sea and Shore Seminars, 2100 19th St, NW, No. 601, Washington, DC 20009 (Attn: N. Chamberlain).

Symposium on "Biological Products in the Treatment of Cancer"

London, England, April 13–15, 1977

This is an open symposium to be held by the International Association of Biological Standardization. The topics to be presented are: principles governing the use of biologic preparations in the treatment of cancer; materials (bacillus Calmette-Guérin, *Corynebacterium parvum*, interferon, etc); production; quality control; mode of action; immunology; pharmacology; experimental oncology; toxicology in animals; clinical relevance of experimental data and design of trials; and clinical studies.

Persons interested in presenting papers at this symposium are asked to submit the proposed title to the Organizing Secretary before December 31, 1976. *Further information and registration forms may be obtained from:* Dr. A. H. Griffith, Organizing Secretary, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England.

Availability of Interferon

In order to foster increased research on interferon in relation to cancer, the National Cancer Institute, National Institutes of Health, Bethesda, Md, is in the process of obtaining a supply of human leukocyte, human fibroblast (diploid) cell, and human lymphoblastoid cell interferons. This material will be made available to qualified scientists who have obtained support for their research but are in need of interferon. The human leukocyte and fibroblast interferons are being prepared in a manner which will make them usable for clinical studies.

Scientists interested in obtaining some of this material should submit their requests to the Interferon Working Group, NCI, NIH, which will be responsible for the distribution of this material. The request should specify the type and amount of interferon requested and should include a complete description of the study for which the interferon is needed and the source of support. If it is to be used in a clinical study, the clinical protocol should also be included.

It should be stressed that limited amounts are available and only the most scientifically deserving proposals will be considered. Therefore, it is to the applicant's advantage to be complete.

Requests should be addressed to: Chairman, Interferon Working Group, NCI, NIH, Bg 31, Rm 3A03, Bethesda, Md 20014.

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